

CHARACTERIZATION AND COMPARISON OF URINE AND SERUM PROTEINS
WITH RENAL BIOPSY FINDINGS AND CLINICAL DATA IN DOGS WITH
NATURALLY OCCURRING PROTEINURIC RENAL DISEASES

A Dissertation

by

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ABSTRACT

Chronic kidney disease (CKD) results in significant morbidity and mortality in dogs, and urine protein loss is common in dogs with CKD. Currently available non-invasive biomarkers for evaluating CKD in dogs cannot accurately predict the severity of glomerular and tubulointerstitial (TI) damage or the cause of CKD without renal biopsy. Non-invasive indicators of degree of renal damage, disease type, and prognosis would be ideal for clinicians and owners. Study goals were to evaluate novel protein biomarkers and electrophoretic banding patterns as indicators of glomerular and TI damage, specific disease type, and survival in dogs with naturally occurring proteinuric CKD. These retrospective studies used urine, serum, and renal biopsies from 200+ dogs with CKD. Selected urinary protein biomarkers (immunoglobulins G and M, retinol binding protein, neutrophil gelatinase associated lipocalin, and N-acetyl- β -D-glucosaminidase (NAG)) and electrophoretic urinary protein banding patterns were evaluated and correlated with histologic damage on renal biopsies, and significant associations, sensitivities, and specificities of biomarkers for renal disease type were determined. The odds of altered urinary biomarkers and banding patterns being associated with increased risk of death due to renal disease were also determined.

Fractional excretions of immunoglobulin M (IgM) and immunoglobulin G correlated most strongly with glomerular damage based on light microscopy, while serum creatinine correlated most strongly with TI damage. Urinary IgM and NAG were significantly associated with immune complex-mediated glomerulonephritis (ICGN),

and urinary IgM in particular had high specificity for ICGN above 13.6 ng/ml or 14.4 µg/mg. Electrophoretic protein banding patterns had excellent sensitivity and specificity for detection of glomerular damage and good sensitivity and excellent specificity for detection of TI damage, and banding patterns were moderately correlated with histologic severity of glomerular and TI damage. Increases in most protein biomarkers, degree of severity of electrophoretic protein banding patterns, and degree of histologic glomerular and TI damage were significantly associated with an increased risk of death due to renal disease.

Novel urine biomarkers and electrophoretic protein banding patterns are useful for detection of glomerular and TI damage, prediction of specific disease types (in particular, ICGN), and prediction of risk of death in dogs with proteinuric CKD.

DEDICATION

This thesis is dedicated to the X-linked hereditary nephropathy colony dogs housed at Texas A&M University, in particular, Avon, Bralyn, Hedwig, Kaney, Lilac, Nymph, and Zita. These research dogs have advanced our knowledge of chronic kidney disease to the benefit of veterinary and human medicine alike.

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The statistical data analyzed in Chapter II was provided by Dr. May Boggess and portions of the statistical analyses in Chapter IV were provided by Dr. Irina Gaynanova of the Department of Statistics at Texas A&M University. The histologic analyses and scoring systems depicted in Chapters II-IV were performed by Dr. Rachel Cianciolo.

All other work conducted for the dissertation was completed by Dr. Hokamp. Laboratory assistance for biomarker measurements and gel electrophoresis was provided by Dr. Marselle Kovarsky, Dr. Marta Gruarin, and Chelsea Retzloff during their summer research fellowships and Sidney Leidy during her time as a student worker.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

Chronic Kidney Disease in Dogs

Chronic kidney disease (CKD) occurs when one or both kidneys are structurally or functionally impaired for a minimum of 3 months.¹ CKD leading to renal failure is a common cause of morbidity and mortality in dogs in which it is often a result of primary glomerular disease.^{2,3} Regardless of the initiating cause of CKD, progressive renal injury is characterized by interstitial fibrosis, peritubular capillary loss, and destruction of functional nephrons, and renal function most closely correlates with the degree of tubulointerstitial (TI) damage.⁴ Structural and functional evidence of kidney damage is prevalent, even in apparently clinically healthy dogs, and prevalence of damage increases with age, approaching 50-90% in some studies.⁵ However, most currently available, non-invasive clinical methods for detecting TI damage and fibrosis are relatively insensitive. In addition, specificity of these tests can be quite poor. There currently is no clinically available non-invasive marker that is sensitive and specific enough to detect ongoing tubular damage, decreased tubular function, and/or progressive fibrosis that ultimately leads to end-stage renal disease. Therefore, although TI damage is frequent in dogs,^{6,7} lesions often are not recognized clinically until they are at an

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advanced stage, when lesions are both severe and irreversible and options for successful therapy are limited. Furthermore, early treatment can help prolong survival in dogs with CKD; therefore, testing strategies that can better identify progressive disease at an early stage would improve a clinician's ability to provide appropriate therapy in a timely fashion. The addition of these tests during serial monitoring may also improve a clinician's ability to more appropriately adjust the treatment plan for each patient.

Renal Proteinuria

Establishing the Presence of Renal Proteinuria

The studies included in this dissertation focus on proteinuric CKD in dogs, particularly those due to primary glomerular diseases. Proteinuria is defined by the detection of an excessive amount of protein in the urine by means of semiquantitative tests on urinalysis (dipstick) or quantitative measurements of urine protein:creatinine (UPC) or urinary albumin concentration.⁸ The origin of proteinuria (renal, pre-renal, or post renal) as well as its persistence and magnitude must be established.⁸ Localizing the source of proteinuria and ruling out pre-renal proteinuria (e.g., due to an overload of hemoglobin, myoglobin, or Bence Jones proteins) or post-renal proteinuria (e.g., due to lower urinary tract infection, inflammation, or hemorrhage) is done by careful review of patient history, physical examination, and evaluation of hematology, serum/plasma biochemistry, and urinalysis findings.^{8,9} Persistent renal proteinuria (UPC \geq 0.5 in at least three samples two or more weeks apart without a contributing pre-renal or post-renal cause) could be glomerular, tubular, or both. Once proteinuria is deemed to be

persistent and renal in origin, evaluation and monitoring of the UPC are important steps in determining the presence and severity of glomerular damage. A persistent UPC between 0.2 and 0.5 is classified as borderline proteinuria while $\text{UPC} \geq 0.5$ is considered proteinuric (<http://www.iris-kidney.com/guidelines/grading.html>). UPC values ≥ 2 are generally considered to be indicative of glomerular proteinuria, while values < 2 are thought to occur more often with tubular proteinuria.^{8,10} Certainly, in the author's experience, a $\text{UPC} \geq 2$ is typically associated with at least some injury to the glomerular filtration barrier, with significant glomerular lesions identified in 99.6% of 501 renal specimens from dogs biopsied for the clinical indication of proteinuria, typically with a $\text{UPC} \geq 2$.¹¹ However, a proportion of proteinuric dogs with $\text{UPC} < 2$ have primary glomerular damage discovered on renal biopsy.¹² Conversely, a small proportion of dogs with $\text{UPC} > 2$ have primary TI damage. Therefore, the magnitude of the UPC cannot always localize renal proteinuria as primary glomerular or TI damage.

Renal proteinuria is commonly observed in dogs with kidney disease; however, the role of proteinuria in canine CKD is likely under-appreciated because affected dogs are often identified late in their disease course. Despite consensus statements and reviews about the importance of evaluating, monitoring, and treating for renal proteinuria, proteinuria is often still overlooked in small animals as an early marker of kidney disease.^{8,9,13,14} In some cases, this may lead to clinicians not detecting renal disease until development of azotemia, missing the opportunity for timely therapeutic intervention.

Pathophysiology of Renal Proteinuria

Glomerular Permselectivity

In the healthy kidney there are several mechanisms that prevent protein loss into the urine. The glomerular filtration barrier, composed of the fenestrated endothelium and glycocalyx, trilaminar glomerular basement membrane (GBM), and podocytes with slit diaphragms, is the main mechanism for preventing proteinuria.¹⁵ The glomerular capillary wall restricts passage of proteins from the blood into Bowman's space on the basis of their molecular size, electrical charge, and sterical configuration. This discrimination of molecules is known as glomerular permselectivity and allows easier passage of small and neutral or positive proteins compared with large and negatively charged proteins.^{15,16}

Proteins must pass through the glomerular wall barriers, the first of which are the open pores of the glomerular endothelium. This is followed by the glomerular basement membrane, which is a collagenous network composed of type IV collagen, laminin, nidogen, and proteoglycans (e.g., chondroitin sulfate proteoglycan and heparin sulfate proteoglycan). The heparin sulfate proteoglycan imparts charge selectivity to the glomerular basement membrane.¹⁶ Finally, proteins must pass through the filtration slits. These are formed by foot processes of podocytes which are negatively charged (due to a surface coat of acidic glycoproteins) and interdigitate to form the slit diaphragm.^{15,16} The fenestrated endothelium acts as an electrostatic barrier for negatively charged proteins. The glomerular basement membrane can restrict movement of large plasma proteins and negatively charged proteins. The most selective barrier for most proteins is considered to

be the slit diaphragm, which is composed of several proteins that create a zipper-like filter. These proteins include nephrin, Neph1, Zona Occludens-1 (ZO-1), P-cadherin, catenins, CD2AP, and podocin. Nephrin and Neph 1 interact with each other to form the backbone of the slit diaphragm. These 2 proteins interact with intracellular adapter proteins podocin, CD2AP and ZO-1 that connect the slit diaphragm to the actin cytoskeleton of the podocyte foot processes.^{15,16} The basal side of the podocyte is attached to the glomerular basement membrane with $\alpha3$ - $\beta1$ integrin and the dystroglycan complex. $\alpha3$ - $\beta1$ integrin forms bonds with the actin cytoskeleton of the podocyte, while the dystroglycan complex is important for proper spacing of matrix proteins resulting in appropriate porosity and permeability of the glomerular basement membrane.¹⁵

In health, plasma proteins with a molecular weight < 40 kilodaltons (kDa) (low molecular weight (LMW) proteins) can freely pass through the glomerular filtration barrier to arrive at the tubular lumen (see below). Intermediate molecular weight (IMW) proteins, those approximately the size of albumin, face increased charge and size restrictions, and high molecular weight (HMW) proteins (> 100 kDa) are generally completely restricted due to their large size.^{15,17,18}

Protein Handling by Renal Tubular Epithelial Cells

In the healthy kidney, LMW proteins and albumin are filtered through the glomerular filtration barrier in significant amounts; however, very few of these proteins are retained in the final urine product. This is because the proteins are reabsorbed from the tubular fluid into the tubular epithelial cells and degraded in lysosomes. This is

followed by exocytosis of peptide products (from protein breakdown) back into the urine. Reabsorption of proteins is via receptor-mediated endocytosis, which involves 2 major proteins, megalin and cubulin.^{15,16,18} These proteins are cooperative and relatively non-selective, as many proteins are able to bind to them.¹⁵ After binding to the receptors, albumin or other proteins are directed into coated pits for endocytosis, which might involve the protein amnionless. The proteins are then transferred to a lysosome and are degraded while the receptors are recycled.^{15,16}

Glomerular and Tubular Damage

When renal damage occurs, the mechanisms that prevent proteinuria are compromised. Glomerular damage increases the permeability of the filtration barrier, allowing increased filtration of IMW and HMW proteins.¹⁵ Tubular damage results in decreased protein reabsorption, leakage of proteins from damaged tubular epithelial cells, and upregulation of proteins involved in injury and repair.^{15,17} Glomerular damage often results in massive proteinuria whereas tubular damage is thought to result in mild proteinuria. Further discussion of the magnitude and patterns of proteinuria that occur with glomerular and TI damage will be found later in this introduction.

Patterns of Proteinuria

Few studies have analyzed urine protein banding patterns in dogs with renal injury using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), sodium dodecyl sulfate agarose gel electrophoresis (SDS-AGE) or similar methods.¹⁹⁻²⁶

Several of these studies have confirmed the limited number of protein bands in non-proteinuric dogs, with primary urinary proteins in healthy dogs consistent with albumin and Tamm-Horsfall Protein.¹⁹⁻²⁵ These studies have also established glomerular, tubular, and mixed banding patterns to determine if glomerular damage, tubular damage, or both are contributing to the proteinuria.^{15,26} Primary tubular damage with limited or no glomerular damage generates a pattern of predominantly LMW protein bands, while primary glomerular damage with minimal or no tubular damage generates a pattern of IMW and HMW protein bands.^{15,19,21,26} Mixed patterns with protein bands in LMW, IMW, and HMW ranges, however, are the predominant patterns seen in proteinuric dogs, as concurrent damage to both glomerular and tubular components commonly occurs.^{19,21,26} Figure 1 demonstrates different urine protein banding patterns from variably proteinuric dogs evaluated by the International Veterinary Renal Pathology Service (IVRPS).

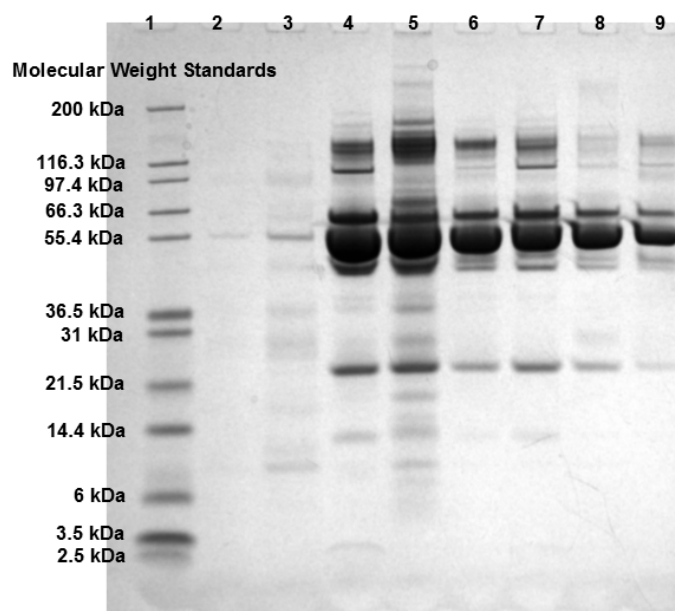


Figure 1. Image from Bis-Tris gel electrophoresis demonstrating urine protein banding patterns obtained from dogs with primary tubular damage (Lanes 2 and 3: low magnitude of proteinuria with predominantly low molecular weight bands), primary glomerular damage (Lanes 6, 7, 8 and 9: relatively large magnitude of proteinuria with predominantly intermediate and high molecular weight bands and few low molecular weight bands), and mixed glomerular and tubular damage (Lanes 4 and 5: mixture of prominent bands ranging from low to high molecular weight).²⁷

While in some studies urine protein banding patterns have correlated well with histologic findings in dogs with renal disease,^{20,21} others have found that urine gel electrophoresis has good sensitivity but poor specificity for detection of glomerular and TI damage in comparison to biopsy.^{19,26} Of interest, presence of particular LMW protein bands appear to be associated with more severe renal disease in dogs, with LMW protein bands at 12 or 15 kDa being 100% specific for advanced TI damage in dogs.²⁶ Along these lines, in humans with primary glomerulonephritides with normal renal function at

study entry, LMW band patterns with bands as low as 10 kDa were significantly associated with development of chronic renal failure and shorter time to end-stage renal disease.²⁸ Furthermore, protein banding patterns were predictive of response to therapy in humans, which has not been assessed in dogs.²⁸

Urine protein patterns on SDS-AGE were also found to have high sensitivity but poor specificity for detection of dogs that are borderline proteinuric. While the authors concluded that SDS-AGE could misclassify the degree of proteinuria when compared with UPC, they did not normalize the urine samples to the concentration of the urine prior to running on the gel, confounding interpretation.¹⁹ These findings demonstrate the ability of gel electrophoresis to detect small amounts of protein in the urine and the need to appropriately normalize samples to accurately interpret results. In Chapter IV, the protocol used in our laboratory to normalize urine samples prior to electrophoretic analysis is discussed.

Protein banding pattern analysis has proven useful to clarify location of renal lesions in clinical studies of dogs with various nephropathies. Indeed, SDS-AGE was used to characterize the origin of proteinuria in clinically healthy Dogue de Bordeaux dogs, a breed with a familial glomerulonephropathy in which many clinically healthy dogs develop proteinuria.²⁰ It was determined that the nephropathy was likely of glomerular origin, and in healthy dogs with proteinuria or borderline proteinuria, SDS-AGE was deemed a good screening tool to rule out glomerular lesions in Dogue de Bordeaux dogs with consistently borderline proteinuria. Urine from dogs with infectious etiologies that can lead to nephropathies, including leishmaniasis, leptospirosis, and

pyometra, have also been evaluated by SDS-PAGE.²³⁻²⁵ Leishmaniasis was shown to generate a predominantly mixed protein pattern, which matched the presence of glomerular and tubular lesions on renal histopathology. Leptospirosis resulted in a predominantly tubular pattern of injury while pyometra resulted in a pattern of predominantly glomerular damage with lesser degree of TI damage.

Although urine protein banding patterns can demonstrate glomerular and tubular damage, several factors can interfere with the interpretation of results. Perini et. al. explained that some LMW bands might not represent true tubular bands, as immunoblotting has demonstrated breakdown products of albumin and immunoglobulins in the LMW regions on SDS-PAGE.²⁹ An increased proportion of 25 kDa proteins were indeed noted in the urine of dogs with leishmaniasis, babesiosis, and ehrlichiosis on non-reducing SDS-PAGE, and the authors hypothesized that these proteins were free immunoglobulin light chains.²⁶ Intact male dogs have repeatedly demonstrated at least one LMW protein (often between 25 – 30 kDa) that is consistent with arginine esterase, a prostatic protein.^{20,30} Finally, although not previously reported in canine studies, presence of hemorrhage or urinary tract infections can introduce protein banding patterns in the glomerular and tubular protein regions that are not seen in healthy dogs with inactive urine sediments (author observations: JAH). Thus, while urine protein banding patterns can contribute to a better understanding of renal lesion localization, other factors can potentially alter correct interpretation. A more thorough discussion of banding pattern interpretation and interferences is provided in Chapter IV.

Urinary Biomarkers of Kidney Disease

While measurement of the UPC is an important first step in the evaluation of renal proteinuria, its limitations include low specificity for determining the source of proteinuria and the severity of injury. Therefore, several specific urine proteins are being investigated as markers of glomerular or tubular damage.

When protein biomarkers are quantified in urine, their concentration is often indexed to urine creatinine (e.g., urinary biomarker divided by urine creatinine) or urine specific gravity. Indexing urinary biomarkers to urine creatinine assumes that the excretion of urine creatinine is constant between and within individuals, and that both urinary biomarkers and creatinine are inversely proportional to urinary flow rate. When these assumptions are met, an increased or decreased biomarker/creatinine ratio will reflect increased or decreased biomarker excretion.³¹ However, when an animal is not in steady state (i.e., when renal function is rapidly changing), the assumption of constant urine creatinine excretion may not be correct, as demonstrated in a study of human patients with acute kidney injury (AKI) and post-kidney transplantation.³¹ Because of this, the practice of indexing to urine creatinine in cases of AKI is questionable. Therefore, some authors have reported concentrations of biomarkers without indexing to creatinine. For purposes of this dissertation, biomarkers that are indexed to urine creatinine are denoted as uBiomarker/c, and those that are un-indexed concentrations in the urine are denoted as uBiomarker.

Most biomarkers that will be discussed in depth in this introduction are those that were measured in dogs for the studies included in this dissertation. However, multiple

other biomarkers have been evaluated in many domestic species, and therefore, Tables A-1, A-2, and A-3 in Appendix A provide an overview of urinary and serum/plasma biomarkers in domestic veterinary species.

Markers of Glomerular Damage/Dysfunction

Immunoglobulins

Immunoglobulins are large glycoproteins made by plasma cells in the spleen, lymph nodes, and bone marrow and are involved in antibody-mediated defense.^{15,32} The molecular weight of immunoglobulins G (IgG) and M (IgM) are 150 kDa^{15,32,33} and 900 kDa,^{15,32} respectively. Serum immunoglobulin A (IgA) is present in monomeric, dimeric, and polymeric forms, with the monomeric form having a molecular weight of 160 kDa.³⁴ Thus, IgG, IgM, and IgA are HMW proteins that cannot pass through the glomerular filtration barrier in the healthy kidney. However, with glomerular damage, they may pass into the urinary filtrate; thus, they are considered markers of glomerular damage.¹⁵

Measurement and Stability

Species-specific ELISAs are the most common and preferred method for detection of urinary immunoglobulins;^{35,36} however, thus far, detection of IgA in canine urine has only been reported using Western blot.²³⁻²⁵ Canine specific ELISA assays for IgG, IgM, and IgA are available (Bethyl Laboratories, Montgomery, TX and Immunology Consultants Laboratories (ICL), Portland, OR) and IgG and IgM ELISA assays have been validated in canine urine and serum showing acceptable mean

intraassay and interassay variabilities,³⁵⁻³⁸ spiking recovery,^{36,37} and dilutional linearity.^{36,37} True stability testing of IgG in urine has not been evaluated; however, uIgG/c in canine urine samples stored for 8 years at -80°C were similar to those stored for 2 years at -80°C.³⁷

Values in Healthy Animals

Mean urinary IgG/creatinine (uIgG/c) is generally < 3 mg/g, with maximum values < 10 mg/g observed in all but one study (Table A-1).^{35,37,39-43} Published studies currently are not available describing the urine concentration of IgM in healthy animals; however, in the author's personal experience, urinary IgM concentration is low in clinically healthy dogs. IgA was undetectable in the urine of healthy dogs using Western blot (Table A-1).²³⁻²⁵

Non-Renal Influences

A few studies found that urinary IgG concentration is not significantly altered by hematuria/hemoglobinuria^{35,42,43} or pyuria/urinary tract infection.³⁵ However, in the author's experience in dogs with primarily proteinuric CKD, uIgG/c was significantly higher in dogs with hematuria, and fractional excretion of IgG (IgG_FE) was higher in dogs with pyuria/bacteriuria compared with dogs with inactive urine sediment (Table A-4; unpublished data: JAH, MBN). Similarly, urinary IgM/creatinine (uIgM/c) and fractional excretion of IgM (IgM_FE) were both significantly increased with hematuria and pyuria/bacteriuria in these dogs (Table A-4; unpublished data: JAH, MBN).

Acute Kidney Injury (AKI)

Urinary IgG is the main immunoglobulin evaluated in diseases known to cause AKI, with fewer studies evaluating urinary IgA. Dogs with AKI due to a variety of causes, including *Babesia rossi*, leishmaniasis, and leptospirosis, have demonstrated increases in uIgG/c^{35,39,40} or IgG and IgA on Western blot,^{23,24,35,39,40} supporting glomerular damage. However, as expected, canine leptospirosis resulted primarily in increased LMW proteins on sodium dodecyl sulfate polyacrylamide gel electrophoresis, consistent with interstitial nephritis.²⁴ Interestingly, in dogs with snake envenomation, UPC was not increased despite significantly increased uIgG/c vs. control dogs at baseline and 24 h later.⁴⁰

Pyometra can cause significant increases in uIgG/c and UPC, with a positive correlation between UPC and uIgG/c.^{25,41,42} This increase is typically transient, decreasing significantly after ovariohysterectomy, and in some cases, uIgG/c returns to values that are not significantly different from healthy dogs.⁴¹ A low proportion of bitches with pyometra also had detectable urinary IgA.²⁵ These studies support that altered glomerular permselectivity can be present in dogs with pyometra, and indeed, histopathology demonstrated glomerulosclerosis as the most common glomerular lesion in these dogs. However, tubular atrophy, interstitial inflammation, and fibrosis were often present as well, and the role of pyometra vs. previous underlying renal disease in these older dogs is uncertain.⁴²

Currently there are no studies evaluating urinary IgM in companion animals with AKI.

Chronic Kidney Disease (CKD)

Urinary IgG^{36,37,44,45} and IgM³⁶ have been shown to increase in dogs with CKD. In dogs with CKD due to X-linked hereditary nephropathy (XLHN), uIgG/c increased in early stages of renal disease while uIgG/c remained low in healthy age-matched littermates.^{37,45} uIgG/c often increased before UPC and continued to increase in mid to late stages of disease progression.³⁷ Furthermore, uIgG/c was moderately to highly positively correlated with most glomerular and tubulointerstitial (TI) lesions based on histopathology.³⁷ Urinary biomarker concentrations and their fractional excretions were measured in dogs with naturally occurring CKD to correlate biomarkers with types of renal damage (glomerular versus TI) and their association with survival.³⁶ Immunoglobulin G (uIgG/c and IgG_FE) and IgM (uIgM/c and IgM_FE) demonstrated moderate, positive correlations with glomerular damage based on light and electron microscopy ($r = 0.44 - 0.58$ and $r = 0.41 - 0.58$), respectively), which were similar to that observed for UPC ($r = 0.45 - 0.57$). Immunoglobulin M_FE also correlated moderately well with TI damage ($r = 0.49$). Markedly increased uIgM/c and uIgG/c were associated with immune complex mediated glomerulonephritis (ICGN), while lower uIgM/c was observed in juvenile nephropathies, non-immune complex mediated glomerulonephropathies, and primary tubular disease. Both IgM_FE and IgG_FE were significantly associated with faster time to death due to renal disease in these dogs.³⁶ The findings from this study are discussed further in Chapter II of this dissertation.

Urinary IgG has been evaluated in several studies of dogs with increased cortisol due to either exogenous or endogenous sources, since excess cortisol causes proteinuria, likely by altering the glomerular filtration barrier. In aged Beagles treated over 24 weeks with hydrocortisone, uIgG/c and UPC progressively increased, while tapering and cessation of hydrocortisone treatment resulted in decreased uIgG/c and UPC.³⁸ In dogs with hyperadrenocorticism, uIgG/c was significantly higher than in clinically healthy controls, supporting glomerular dysfunction.⁴³ Finally, in dogs treated with trilostane or hypophysectomy for ACTH-dependent hyperadrenocorticism, uIgG/c decreased up to 15 fold post-treatment. However, uIgG/c did not completely return to levels comparable to healthy dogs in all cases, with persistence of proteinuria in 38% of dogs 12 months post-treatment.⁴⁶

Additional Urinary Proteins Indicating Glomerular Damage/Dysfunction

Albumin

The use of urinary albumin in dogs as a renal biomarker has recently been reviewed.⁴⁷ Albumin is a negative acute phase, IMW (approximately 65 kDa) serum protein synthesized primarily by hepatocytes. It is considered primarily to be a marker of glomerular damage, but urinary albumin can also be present with tubular or vascular damage.^{14,15,48,49} Measurement of urinary albumin has been validated in dogs,⁵⁰ and urinary albumin/creatinine is elevated in both AKI and CKD,^{44,45,50,51} including renal damage due to nephrotoxic drugs (methyl cantharadimide tablets and gentamicin),^{52,53} snake envenomation,⁴⁰ pyometra,^{41,42} and hypercortisolism.^{38,43,46} Pyometra caused

transient albuminuria that significantly decreased or returned to values not significantly different from healthy dogs after ovariohysterectomy.^{41,42}

Markers of Tubular Damage/Dysfunction

The proteins discussed in the next section are abnormally present in the urine due to decreased tubular reabsorption (retinol binding protein [RBP], neutrophil gelatinase-associated lipocalin [NGAL], cystatin C), upregulation of proteins involved in injury and repair (NGAL), and decreased production by damaged tubules (Tamm-Horsfall protein [THP]). Proteins present due to release from damaged tubular epithelial cells are discussed in the urinary enzyme section. Additional examples of urinary biomarkers present due to these 4 mechanisms are presented in Table A-2.

Retinol-Binding Protein (RBP)

Retinol-binding protein (RBP) is a 21 kDa lipocalin that acts as the transport protein for retinol in plasma.⁵⁴ Retinol-binding protein is primarily produced in the liver but also in the kidney, lungs, spleen, brain, stomach, heart, and skeletal muscle.⁵⁵⁻⁵⁷ Retinol-binding protein circulates in a complex with transthyretin (TTR), which has a molecular weight of 54 kDa.^{54,58,59} By itself, RBP is a LMW protein that can freely pass through the glomerular filtration barrier; however, the TTR-RBP complex is too large to pass through the glomerular filtration barrier in the healthy kidney.⁵⁸ Retinol-binding protein in the renal filtrate is reabsorbed by tubular epithelial cells.⁶⁰ Tubular damage and/or competition for reabsorption by the presence of abnormally large amounts of

protein (i.e., with glomerular damage) results in decreased reabsorption of RBP with subsequent loss of RBP into the urine.^{61,62} Glomerular damage could also contribute to urinary RBP due to loss of the TTR-RBP complex.

Measurement and Stability

Human RBP immunoassays have been validated for dogs with adequate intraassay and interassay variabilities (canine urine and plasma),^{35,37,63} spiking recovery (canine urine),³⁷ and dilutional linearity along a specific range of the standard curve (canine urine).^{35,37} A canine-specific RBP ELISA was recently marketed (ICL); however, validation and use of this kit has not yet been published. Retinol-binding protein concentration is similar in cystocentesis vs. voided urine samples from clinically healthy dogs.⁶⁴ Retinol-binding protein appears to be relatively stable in canine urine samples when frozen, ideally at -80°C.^{37,64} All but one study⁶⁴ normalized urinary RBP concentration to urinary creatinine (uRBP/c [mg/g]).^{35-43,46,50,51,65-68}

Values in Healthy Animals

In healthy dogs, RBP is generally undetectable or minimally detectable in the urine by Western blot.^{69,70} Using immunoassays to quantify urinary RBP in healthy dogs, the highest reported uRBP/c was 0.9 mg/g,⁴³ with most studies reporting means and medians < 0.15 mg/g regardless of assay used (Table A-2).^{35,37-43,46,50,51,65}

Non-Renal Influences

In dogs, age does not appear to be a major influence on uRBP/c, as no significant differences were found for uRBP/c between healthy young and older dogs.^{37,50} However, a mild but statistically significant negative correlation with age was found in young adolescent dogs, likely due to low creatinine excretion in very young dogs.³⁷ Pyuria, bacteriuria or positive urine culture, and at least mild to moderate hematuria and hemoglobinuria do not seem to significantly affect uRBP/c;^{35,39,64} however, in one study, a mild increase in uRBP was seen in markedly hematuric samples.⁶⁴ In the author's experience, RBP_FE was significantly higher in pyuric/bacteriuric samples versus inactive sediments from dogs with proteinuric CKD (Table A-4; unpublished data: JAH, MBN).

Renal Disease in Veterinary Medicine

AKI

Naturally occurring AKI (e.g., pyometra, babesiosis due to *Babesia rossi*, and envenomation by cytotoxic and neurotoxic snakes) transiently increases uRBP and uRBP/c in dogs, presumptively indicating tubular dysfunction.^{35,40,42,69} Histologic confirmation of tubular damage in dogs with pyometra found that dogs with severe TI lesions on histopathology had higher uRBP/c (in the 75th percentile) compared with dogs demonstrating milder lesions.⁴² Typically, uRBP/c decreased significantly after ovariohysterectomy in these dogs, often to values comparable to healthy dogs.^{35,42}

CKD

More studies have evaluated urinary RBP for detection of tubular dysfunction in dogs with CKD than with AKI, and dogs with CKD have significantly increased uRBP,⁶⁹ uRBP/c,^{35,37,44,45,50,51,63,70} and RBP_FE^{51,63} compared with healthy dogs. However, whether this increase is primarily present due to tubular damage as opposed to presence of proteinuria is controversial, as discussed below.

In dogs with CKD due to XLHN, uRBP/c was increased prior to onset of azotemia but after onset of proteinuria, and urinary RBP increased with disease progression throughout all disease stages (based on sCr), with the most pronounced increase in mid to late stages of renal disease.^{37,45,70} Furthermore, uRBP/c had the strongest correlation with both glomerular and TI lesions compared with other biomarkers of renal function, and it correlated most strongly with conventional measures of disease severity (sCr, glomerular filtration rate (GFR), and interstitial fibrosis).³⁷ Despite this, uRBP/c was not a significant independent predictor of GFR based on multivariate analysis. It was concluded that uRBP/c might be useful for detecting early tubular damage before an obvious increase in sCr.³⁷

When biomarkers were correlated with histologically proven renal damage in dogs with naturally occurring CKD due to a variety of causes, uRBP/c and RBP_FE were moderately correlated with glomerular and TI damage, with RBP_FE having the second strongest correlation with TI damage following sCr.³⁶ RBP_FE also increased significantly with each increase in IRIS stage, while uRBP/c only increased significantly in IRIS stages 3 and 4. Both uRBP/c and RBP_FE were significantly associated with

time to death due to renal disease when evaluated individually; however, in a multivariate analysis with other biomarkers, neither uRBP/c nor RBP_FE were significantly associated with survival.³⁶ These results are discussed in greater depth in Chapter II of this dissertation.

Despite the promise of urinary RBP for early detection of CKD and monitoring of progression, another study concluded that proteinuria influenced uRBP/c more than decreased renal function based on sCr and plasma creatinine clearance. This conclusion was based on finding uRBP/c to be significantly greater in dogs with proteinuria and borderline proteinuria compared with azotemic, non-proteinuric dogs and the inability of uRBP/c to detect reduced GFR.⁵¹

Dogs with hyperadrenocorticism had higher uRBP/c compared with control dogs; however, UPC was also significantly higher in these dogs compared to controls.⁴³ Additionally, in dogs with ACTH-dependent hyperadrenocorticism, uRBP/c decreased significantly after treatment with hypophysectomy,⁴⁶ such that post-treatment median uRBP/c values were within the range reported for healthy dogs. Urinary RBP/c also decreased after treatment with trilostane; however, the decrease was not significant, and several dogs had persistent proteinuria.⁴⁶ These cumulative results suggest that ACTH-dependent hyperadrenocorticism results in decreased tubular protein reabsorption; however, the degree of reversibility and post-treatment values for uRBP/c depend on the type of treatment (hypophysectomy versus trilostane) and persistence of proteinuria.⁴⁶ Finally, in aged dogs treated with hydrocortisone, uRBP/c increased with treatment and decreased post-treatment.³⁸

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) is a member of the family of lipocalin binding proteins originally isolated from the specific granules of human neutrophils,^{71,72} but it is also present in many normal tissues including the kidney.⁷³ Neutrophil gelatinase-associated lipocalin is upregulated in epithelial cells in neoplastic^{73,74} and inflammatory⁷⁴ processes. The original proposed function of NGAL was as a bacteriostatic agent that binds bacterial siderophores to prevent iron acquisition.⁷⁵ Neutrophil gelatinase-associated lipocalin has since been found to also be involved in many cellular mechanisms including renoprotection.⁷⁶ Neutrophil gelatinase-associated lipocalin is a LMW protein that freely passes through the glomerular filtration barrier and is reabsorbed almost completely by the proximal tubules in the healthy kidney.⁷⁶ With either renal damage or protein overload, reabsorption of NGAL in the proximal tubules is impaired. In addition, there is increased synthesis of NGAL by damaged tubular epithelial cells.⁷⁷ Three different molecular weight forms of NGAL are present in canine urine,⁷⁸ including a 25 kDa monomeric protein that appears to originate from renal tubular epithelial cells and is associated with renal damage, a 45-50 kDa dimeric protein that is the predominant form released by neutrophils and appears most often with pyuria, and an NGAL/MMP-9 heterodimer complex that occurs with both renal injury and pyuria and hematuria.^{71,78,79} Although ELISAs that distinguish between different molecular forms of human NGAL are available, canine NGAL ELISAs are currently unable to make this discrimination.⁸⁰

Measurement and Stability

NGAL has been evaluated in urine, serum, and plasma in dogs, and it has been partially validated using canine specific ELISAs with acceptable intraassay and interassay variabilities, dilutional linearity, and spiking recovery in canine urine and plasma.^{36,37,81,82} Neutrophil gelatinase-associated lipocalin has also been validated in canine serum, but results were not published.⁸³ Neutrophil gelatinase-associated lipocalin appears to be relatively stable in canine urine, as there were minimal effects on uNGAL after 4 freeze thaw cycles, and no significant differences in uNGAL/c were observed in samples collected up to 8 years apart, stored at -80°C.³⁷ No difference in uNGAL/c was apparent between cystocentesis and voided samples⁸⁴ or between 2-hour spot urine samples and 15-hour collection samples.⁸⁵ The 3 different molecular weight forms of NGAL can be differentiated in canine urine samples using Western blot.⁷⁸ Values in healthy dogs are reported in Table A-2.

Non-Renal Influences

In one study, healthy dogs < 4 months of age often had much higher uNGAL/c than dogs > 4 months of age, but it was suggested that this was likely due to preputial neutrophil contamination from voided urine samples from the youngest dogs.³⁷ In another study, there was no correlation of uNGAL/c with age in adult dogs, although uNGAL (not normalized) was weakly but positively correlated with age. Furthermore, this same study found that both uNGAL and uNGAL/c decreased with body mass.⁸¹ Interestingly, peripheral WBC count does not correlate with overall serum NGAL

(sNGAL);⁸⁶ however, dogs with monomeric uNGAL had significantly higher peripheral WBC and neutrophil counts than dogs without the uNGAL monomer.⁷⁸

Pyuria and urinary tract infections can markedly influence urinary NGAL concentrations. Several studies showed that both uNGAL and uNGAL/c were significantly higher in dogs with pyuria, urinary tract infections (UTI), and other lower urinary tract diseases (such as urothelial cell carcinoma and calcium oxalate urolithiasis), both with and without azotemia, compared with healthy controls.^{78,81,84} In the author's experience in dogs with proteinuric CKD, uNGAL/c was higher in dogs with active vs. inactive sediments (Table A-4; unpublished data: JAH, MBN). Although dogs with lower urinary tract diseases had increased uNGAL/c compared with control dogs, values were still lower than dogs with renal diseases.^{78,84} A uNGAL/c cutoff of $> 2.57 \mu\text{g/g}$ and a uNGAL cutoff of $> 3.38 \text{ ng/ml}$ had sensitivities and specificities in the 70-80% range for dogs with vs. without a UTI.⁸¹ Furthermore, the 50 kDa dimer form of NGAL was shown to be more common in urine from dogs with pyuria, while the NGAL/MMP-9 complex was found in dogs with pyuria and hematuria.⁷⁸

While one study reported that certain non-renal diseases (gastritis, PLE, hepatic disease, enteritis, portal shunt, bone fracture, intervertebral disk disease) do not appear to affect uNGAL,⁷⁸ uNGAL and uNGAL/c were significantly increased in dogs with carcinoma and lymphoma compared to healthy dogs; however, it was acknowledged that it could not be ruled out that some of the study dogs with neoplasia also had early CKD.⁸⁷ Thus, non-urinary diseases in dogs might increase urinary NGAL in dogs, and if so, this finding would further indicate lack of renal specificity of this biomarker.

AKI

While studies of drug-induced AKI consistently demonstrate increases in urinary NGAL, mixed results are reported with regard to timing of this increase, which could partly be due to differing protocols, particularly with varying concentrations of gentamicin used. Three separate studies in dogs given gentamicin found that uNGAL/c and uNGAL increased early (as early as day 1 post-administration) compared with other markers of nephrotoxicity (such as sCr and urea nitrogen) and was correlated with the severity of tubular damage, including tubular cell necrosis, degeneration and regeneration, tubular cell hyaline droplet formation, and hyaline casts.^{85,88,89} Furthermore, the decrease in uNGAL/c heralded recovery earlier than a decrease in sCr by a median of 2 days.⁸⁹ It was concluded that uNGAL/c was a sensitive and predictive marker of gentamicin-induced nephrotoxicity.^{85,89} However, a fourth study found that although uNGAL/c was significantly correlated with GFR and increased at the first time point evaluated (4 days after gentamicin administration), the increase was not statistically significant until the second time point, at 8 days post administration. Thus, in this study, uNGAL/c was not superior to more traditional markers of renal function.⁵³ In AKI induced by administration of intravenous polymyxin B for 7 days, a dose dependent increase in uNGAL/c was observed, with significantly increased uNGAL/c in the mid- and high-dose groups on day 2.⁹⁰ Finally, Beagle dogs administered methyl cantharidimide tablets over 30 days demonstrated significant increases in uNGAL in the

high dose group, while middle and low dose groups did not have significant changes in uNGAL.⁵²

Repeatedly, studies have shown promise for NGAL as a marker of naturally occurring AKI in dogs. Urinary NGAL (not normalized), uNGAL/c, and plasma NGAL (pNGAL) were all significantly greater in dogs with azotemia from natural causes (whether due to AKI or CKD) versus healthy control dogs,^{78,82,84,86} and extremely high pNGAL differentiated AKI from CKD in one study⁸² while extremely elevated uNGAL/c was seen in dogs with AKI compared to both CKD and lower urinary tract diseases in another study.⁸⁴ Furthermore, uNGAL/c was increased in non-azotemic (IRIS Grade I) AKI, indicating the presence of kidney injury before sCr increased outside of the reference interval.⁸⁴ Similarly, in dogs that developed AKI post-operatively, median uNGAL was significantly greater 12 hours post-surgery and remained elevated for at least 3 days, while sCr did not show a significant increase until 24 hours post-surgery.⁹¹ Urinary NGAL and uNGAL/c were also shown to be significantly increased (average of 24-fold and 41-fold, respectively) from baseline within 2 hours of reperfusion using a hemorrhage and colloid fluid resuscitation model of renal injury in Greyhounds.⁹² Thus far, uNGAL/c, uNGAL, and sNGAL have not been shown to be predictors of survival in dogs with AKI.^{84,86}

CKD

Dogs with CKD have demonstrated increased uNGAL/c, uNGAL, sNGAL, and pNGAL compared with healthy dogs and dogs with UTI or other lower urinary tract diseases.^{37,78,82-84,86,87} Similar to findings in AKI studies, increases in uNGAL/c occurred

early in the development of CKD in dogs with XLHN.³⁷ As will be discussed further in Chapter II, serum NGAL and fractional excretion of NGAL (NGAL_FE) also increased with increasing IRIS stages in dogs with proteinuric CKD.³⁶ Furthermore, uNGAL/c and NGAL_FE correlated moderately to strongly with both glomerular and TI lesions in dogs with CKD.^{36,37} Finally, higher sNGAL and NGAL_FE are associated with shorter survival compared with lower values in dogs with CKD.^{36,86} Serum NGAL concentration was actually concluded in one study to be a better predictor of clinical outcomes than sCr for dogs with CKD that were already azotemic;⁸⁶ however, this was not repeatable in a second study of dogs with CKD that were predominantly proteinuric but included both azotemic and non-azotemic dogs.³⁶

N-acetyl- β -D-glucosaminidase (NAG)

Renal tubular epithelial cells contain enzymes which have been explored as biomarkers of tubular damage (Table A-2). One of the most commonly studied enzymes in domestic animals is N-acetyl- β -D-glucosaminidase (NAG), a lysosomal enzyme.⁹³ Two isoenzymes of NAG occur in the kidney: NAG-A and NAG-B. In people, only NAG-A can be detected in urine from patients without renal disease, whereas renal damage and disease results primarily in increased excretion of NAG-B.⁹⁴⁻⁹⁶ Although present in the serum and other tissues and cells, urinary NAG originates from the renal tubules in the absence of glomerular damage.⁹⁷⁻¹⁰⁰

Measurement and Stability

Activity of urinary enzymes is expressed as units per liter, and an activity index is typically calculated by normalizing to urinary creatinine concentration. Validation of assays for urinary NAG activity (uNAG) has been performed in dogs.^{37,101} In canine urine, overall intraassay and interassay variabilities, dilutional linearity, and spiking recovery for NAG were acceptable.^{37,50,101} However, canine urine samples with lower concentrations of NAG tended to show higher coefficients of variation.³⁷ NAG activity is relatively stable at -80°C for at least one year; however enzyme degradation, which is not prevented by addition of a protease inhibitor, is still possible.^{37,64,101} Studies assessing stability at room temperature, 4°C, and -20°C have shown contrasting findings, although NAG appears to be stable for at least one month at -20°C in canine urine.^{37,64,101} Up to 4 - 5 freeze-thaw cycles have been shown to significantly affect urinary NAG activity in dogs.³⁷ Thus it is recommended to keep urine samples frozen at -80°C and to limit the number of freeze-thaw cycles. No significant difference in uNAG activity was seen between voided and cystocentesis samples from dogs.⁶⁴

Values in Healthy Animals

Urinary NAG is generally present in low levels in healthy domestic animals (Table A-2).

Non-Renal Influences

Age does not appear to affect uNAG/c in dogs.^{64,102} However, 3 out of 4 studies found uNAG/c was significantly higher in males than females.^{101,103-105} Additionally, uNAG/c decreased after castration of male dogs; therefore, the increase in NAG activity in males may be due to the enzyme content of sperm.^{101,104}

Spot measurements of uNAG/c were significantly correlated with 24-hour urine NAG excretion.¹⁰⁶ However, large intra- and inter-individual variability was noted for uNAG/c.¹⁰¹

Hematuria, pyuria, and bacteriuria/UTI without concomitant pyelonephritis does not appear to affect uNAG or uNAG/c in dogs.^{64,105} However, dogs with lower UTIs accompanied by pyelonephritis had markedly increased uNAG/c values, likely indicating the presence of tubular damage.¹⁰⁵ Further studies are needed to determine if uNAG/c can accurately detect pyelonephritis. Additionally, changes in urine pH did not seem to affect uNAG/c in dogs.¹⁰³

Renal Disease in Veterinary Medicine

AKI

NAG has been most extensively studied as an early marker of AKI by chemical (drug) induction in domestic species. Gentamicin, especially high doses, significantly increased uNAG/c in dogs as early as 24 hour post-administration, and uNAG/c continued to increase throughout the study period.^{53,88,106} Another study found that uNAG/c correlated with the severity of renal lesions and had high accuracy and

sensitivity for detection of renal injury compared with urea nitrogen.⁸⁸ These and other studies support that urinary enzymes such as NAG are more sensitive and reliable for detecting acute renal tubular damage induced by gentamicin than markers of GFR.¹⁰⁷

Other nephrotoxic drugs that resulted in early increases in uNAG/c in dogs include polymyxin B⁹⁰ and methyl cantharidimide.⁵² One study reported a marked increase in uNAG/c in 1 dog administered a therapeutic dose of ketoprofen, a non-steroidal anti-inflammatory drug (NSAID), at day 6 of a 30-day study, but the uNAG/c returned to a clinically normal value at day 21 despite continued drug administration until day 30.¹⁰⁸ Thus, administration of ketoprofen at a therapeutic dose did not appear to significantly increase uNAG/c in 4 out of 5 dogs.¹⁰⁸ Additionally, dogs given intravenous polymyxin B for 7 days demonstrated dose dependent increases in uNAG/c, with significantly increased uNAG/c occurring in the mid- and high-dose groups on day 2.⁹⁰

Few studies are available evaluating urinary NAG activity in cases of naturally occurring AKI in domestic animals. Dogs with pyometra have demonstrated increased uNAG/c that, in several studies, decreased back into the range of healthy dogs after ovariohysterectomy, supporting transient AKI in these dogs.^{41,42,102,105} Furthermore, markedly increased uNAG/c was associated with severe TI lesions and reduced GFR in dogs with pyometra.^{42,102}

CKD

Urinary NAG/c appears to be increased in most dogs with CKD as compared to healthy controls.^{37,50,105} In dogs with XLHN, mild increases were observed as one of the

earliest findings, even before increased UPC; however, it did not continue to increase with disease progression beyond mid-stage disease.³⁷ Furthermore, uNAG/c was inconsistently associated with IRIS staging in dogs with proteinuric CKD.^{36,50} Interestingly, in dogs with XLHN, uNAG/c correlated moderately to strongly with both glomerular and TI damage lesions.³⁷ In contrast, uNAG/c at the time of biopsy in dogs with naturally occurring CKD (mostly proteinuric and variably azotemic) correlated moderately well with glomerular damage alone and did not correlate with TI damage, as will be discussed further in Chapter II.³⁶ Additionally, uNAG/c correlated moderately to strongly with UPC and uIgG/c in both studies.^{36,37} These findings support that uNAG/c might be a better indicator of glomerular rather than tubular damage in canine proteinuric CKD.

Endocrine diseases may also influence urinary NAG activity, presumably due to renal damage. Dogs with hyperadrenocorticism had significantly higher uNAG/c compared with control dogs.⁴³ Furthermore, while several other biomarkers (uALB/c, uIgG/c, uRBP/c, and UPC) demonstrated significant decreases after treatment for hyperadrenocorticism, uNAG/c did not decrease significantly even after treatment with trilostane and hypophysectomy.⁴⁶ However, several dogs that were treated for hyperadrenocorticism had persistent proteinuria.⁴⁶ Despite increased uNAG/c in dogs with increased cortisol due to naturally-occurring hyperadrenocorticism, uNAG/c was not increased in dogs treated with hydrocortisone, both in comparison to the control group and within the treatment group itself.³⁸ In dogs with diabetes mellitus, increased uNAG/c was observed when the disease was not controlled (i.e., hyperglycemia,

glucosuria, and ketonuria were present) whereas uNAG/c values were comparable to healthy dogs when blood glucose was controlled.¹⁰⁵

Most studies show that urinary NAG largely trends with proteinuria. A possible reason behind the stronger correlation of urinary NAG with glomerular damage/proteinuria as compared with TI damage/azotemia could be increased lysosomal activity as opposed to active proximal tubular cell damage. However, when glomerular proteinuria is present, it is reasonable that increased uNAG/c at least partially represents loss of NAG from the blood due to altered glomerular permeability.

Tamm-Horsfall Protein

Tamm-Horsfall protein (THP, otherwise called uromodulin) is a 100 kDa protein¹⁰⁹ present in the thick ascending limb of the loop of Henle and the distal convoluted tubule.^{109,110} Tamm-Horsfall protein is one of the major urinary proteins present in healthy dogs.¹⁰⁹ The biologic function of the protein is still not fully understood, but it is believed to have roles in water and electrolyte balance in the thick ascending limb of Henle's loop, defense against urinary tract infections, prevention against the formation of kidney stones, and in innate immunity of the kidney.¹¹¹ Normal urine has high concentrations of THP, and significantly reduced urinary THP concentrations and uTHP/c are seen in dogs with renal disease, including CKD.^{44,63,69,112,113} Furthermore, uTHP/c correlates negatively with plasma creatinine concentration and UPC.¹¹³ Therefore, reduced urinary THP might be a marker of distal tubular damage in dogs and.

Serum Biomarkers of Kidney Disease

As discussed, proteinuria is the earliest marker of glomerular disease, but urine is often not evaluated in many patients for a variety of reasons. Additionally, primary TI disease might not present with clinically evident proteinuria. Therefore, serum biomarkers remain important tools for the diagnosis of kidney disease, serving as endogenous markers of GFR. For decreased GFR to be clinically detectable, substantial losses of nephrons and decreases in overall kidney function are necessary, with estimates of up to 50% or more decrease in function occurring before azotemia develops. However, improved interpretation of sCr can lead to an earlier diagnosis of kidney disease, and new biomarkers of GFR show promise as early indicators of decreased kidney function. It is important to remember, however, that markers of GFR should always be interpreted in light of the urine specific gravity and clinical history, because hydration/blood volume status and urinary tract obstruction can influence these values.

Historically, sCr has been interpreted in the context of a reference interval. Particularly in dogs, where a variety of breeds and sizes are represented, use of a standard, laboratory-based reference interval is an insensitive method for determining whether sCr is increased in a particular patient, given that muscle mass is the major determinant of baseline sCr concentration. More appropriately, a baseline healthy adult measurement would be obtained, followed by monitoring at regular intervals (i.e. during routine yearly health checks). This allows for recognition of small, progressive increases that can indicate relatively large decrements in kidney function. Certainly, CKD can be detected much earlier by such ‘trending’ of sCr, as evidenced in one study in dogs with

CKD due to XLHN, where an average decrease in GFR of approximately 25% was identified compared with 50% when using a reference interval.¹¹⁴ Earlier identification of AKI is also possible using trending, and this is reflected by the IRIS AKI grading guidelines, wherein an increase in sCr of 0.3 mg/dL within 48 hours is used to diagnose Grade 1 or 2 AKI (<http://www.iris-kidney.com/guidelines/grading.html>). Of course, such subtle increases in sCr require excellent laboratory and instrument precision, and this can present a problem, particularly with benchtop instruments commonly used in veterinary clinics.^{115,116} Muscle wasting in a patient over time can also interfere with the ability to identify small, but clinically significant increases in sCr. For instance, one study found cats >15 years old to have lower sCr but also lower GFR than cats <15 years old.¹¹⁷

In an effort to overcome some of the limitations of sCr in evaluation of kidney function, cystatin C and symmetric dimethylarginine (SDMA) have been evaluated as new markers of GFR. Cystatin C is a small protein, while SDMA is a methylated amino acid of similar size to creatinine. In the few published studies on cystatin C in dogs, it correlated as well or better than sCr with measured GFR using clearance techniques in dogs with renal disease, and it was a more sensitive indicator of decreased GFR than sCr.¹¹⁸⁻¹²⁰ Thus far, three studies have shown SDMA to be consistently more sensitive than sCr for detecting decreased GFR in both dogs and cats when sCr was interpreted based on a reference interval.^{117,121,122} It was also more sensitive than sCr based on trending in young, growing dogs,¹¹⁴ possibly because of SDMA's lack of influence by muscle mass.^{117,123} While studies with both of these markers appear promising, they are thus far limited in the scope of non-renal injuries examined. Furthermore, non-renal

influences are still largely unknown, although the major limitation of sCr (muscle mass) does not appear to influence either cystatin C or SDMA. As we gain more experience with these new markers, and in particular, SDMA, we will learn more about their limitations as well as their advantages as compared with sCr.

A summary of serum and plasma biomarkers of kidney disease in dogs and cats can be found in Table A-3.

Study Objectives

Although renal biopsy is certainly the gold standard for evaluating the type of renal disease and severity of damage in dogs, many dogs are not candidates for the procedure due to health or financial reasons. The use of less invasive and inexpensive diagnostic methods, such as urinary electrophoretic protein banding patterns and serum and urinary biomarkers, can provide evidence for the presence or absence of glomerular disease and/or tubulointerstitial disease and might also influence the decision to perform a renal biopsy.

As discussed earlier, Nabity et. al. previously evaluated several urinary biomarkers, including uIgG/c, uRBP/c, uNGAL/c, and uNAG/c in dogs with XLHN and found that each of these biomarkers was moderately to strongly correlated with most of the glomerular and tubulointerstitial lesions that developed during progression of CKD.³⁷ The studies presented in this dissertation focus on evaluation of novel non-invasive tests of renal damage in dogs with naturally occurring CKD in order to expand the work performed in the XLHN dog model of CKD to a cohort of dogs more representative of

those in a veterinary clinical setting. Patterns of proteinuria and specific urinary proteins were evaluated in dogs with a variety of naturally occurring renal diseases to determine their usefulness as markers of renal damage and predictors of specific types of renal diseases and prognosis as compared with conventional clinical tests and the current gold standard, histologic evaluation. The rationale for performing these studies is that conventional non-invasive tests of renal function and damage are unreliable for determining the extent of glomerular and TI injury. Certain urinary tests in people, however, have resulted in improved detection and localization of clinically significant renal injury, and they have provided better severity and prognostic information than standard clinical testing. The urinary analytes evaluated in this investigation have previously been studied either minimally or not at all in veterinary medicine. The studies presented in this dissertation are some of the largest and most extensive to evaluate urinary biomarkers in dogs with naturally occurring CKD to date. The hypotheses in initiating these studies were that certain urinary proteins and protein banding patterns will provide improved detection of glomerular and TI damage and specific disease types, correlation with histologic severity of damage, and prediction of prognosis in dogs with renal diseases as compared with conventional non-invasive tests such as UPC, sCr, and urine specific gravity.

CHAPTER II

CORRELATION OF URINE AND SERUM BIOMARKERS WITH RENAL DAMAGE AND SURVIVAL IN DOGS WITH NATURALLY OCCURRING PROTEINURIC CHRONIC KIDNEY DISEASE*

Introduction

Chronic kidney disease (CKD) is a common cause of morbidity and mortality in dogs,^{124,125} and current non-invasive methods of diagnosis often lack sensitivity, specificity, or both for early disease detection and for identification of the underlying disease process. Clinically, CKD in dogs is typically detected by the presence of renal azotemia, persistent renal proteinuria, or both, often in conjunction with decreased urine concentrating ability, abnormal findings on urine sediment examination (such as presence of casts in the sediment), and abnormal appearance of the kidneys on ultrasound. Persistent renal proteinuria, typically quantified by measuring urine protein:creatinine (UPC), can be an early indicator of CKD in dogs,^{8,124} and it is a negative prognostic factor in dogs with CKD.¹²⁶ However, when mildly increased, UPC cannot differentiate glomerular from tubular damage. Renal biopsy is considered the gold standard for determining type of renal damage,¹²⁷ but it is an invasive procedure and is not feasible in every case due to financial constraints or animal health. Therefore,

* Portions of this chapter are reprinted with permission from Correlation of Urine and Serum Biomarkers with Renal Damage and Survival in Dogs with Naturally Occurring Chronic Kidney Disease by J.A. Hokamp, R.E. Cianciolo, M. Boggess, G.E. Lees, S.L. Benali, M. Kovarsky, and M. B. Nabity, 2016. Journal of Veterinary Internal Medicine 30, 591-601, Copyright 2016 by Wiley Periodical, Inc.

less invasive, inexpensive, sensitive and specific methods to evaluate the presence, character, and severity of kidney damage in dogs are needed.

Urine and serum biomarkers can be useful in human and veterinary medicine for early identification and localization of renal damage and as more sensitive and specific indicators of disease. In proteinuric kidney diseases, differently sized proteins are present in urine secondary to damage to different regions of the nephron (e.g., glomeruli vs. tubules). For example, the presence of high molecular weight proteins, such as immunoglobulins, in urine is indicative of glomerular damage.¹⁵ In contrast, low molecular weight proteins and tubular enzymes are thought to be more specific for renal tubular damage.¹⁵ Of these urine proteins, a few have been recently evaluated in veterinary medicine. Urine immunoglobulin G (uIgG) and urine retinol binding protein (uRBP) were increased in dogs with primary CKD³⁷ and those with renal dysfunction secondary to various systemic diseases including pyometra, babesiosis, and snake envenomation.^{37,39-42,50,51,69,70} Urine, plasma, and serum neutrophil gelatinase-associated lipocalin (NGAL) and urine N-acetyl- β -D-glucosaminidase (uNAG), a renal tubular enzyme, are tubular markers increased in both acute and chronic kidney disease in dogs.^{37,41,42,50,82-86,88,105} NGAL originates not only in the renal tubules, but also from neutrophil granules and many other organs.⁷³ Novel urine biomarkers are not regularly used as diagnostic tools for evaluation of renal disease in veterinary medicine, and few veterinary studies correlate biomarkers with histologically-proven renal damage^{37,42,70,85} and case outcome.¹²⁶

The objective of our study was to determine correlations of promising novel urine biomarkers of renal damage (IgG, immunoglobulin M (IgM), RBP, NGAL, and NAG) with pathologic assessment of glomerular and tubulointerstitial (TI) damage in dogs with naturally occurring, primarily proteinuric CKD due to a variety of causes. Our goal was to determine if the biomarkers provided an indication of the presence and severity of glomerular and/or TI damage, which would support their use as non-invasive tests to detect and monitor proteinuric CKD. We also determined sensitivities and specificities of the biomarkers for detection of specific types of renal disease and evaluated follow-up information from these dogs to determine if the biomarkers might be useful as survival indicators.

Materials and Methods

Sample Collection and Processing

This retrospective study used canine samples of urine supernatant, serum, and kidney tissue collected by the dog's veterinarian and submitted to the International Veterinary Renal Pathology Service (IVRPS) for diagnostic purposes between January 2008 and September 2013. All samples were shipped on ice and were typically received and processed the day following collection. Urine supernatant and serum were aliquoted and stored at -80°C until analysis. Cases were categorized as having inactive urine sediment, an active urinary tract infection (based on culture or sediment findings), hematuria (grossly or microscopically (>100 red blood cells per 40× field)), or pyuria (>10 white blood cells per 40× field), identified either on the submitted sample, if

available, or within 4 weeks of renal biopsy. Cases with an active sediment were excluded from analysis. Renal biopsies were routinely processed for light (LM) and transmission electron microscopy (TEM) as previously described.¹¹ Criteria for diagnosis of renal disease included persistent proteinuria, azotemia, or both. Cases were categorized as having CKD, acute kidney injury (AKI), both CKD and AKI, or not enough information available to determine chronicity of renal disease. CKD was defined by evidence of renal disease for at least 3 months or evidence of chronicity on renal ultrasound or histology.

Histopathological Analysis and Scoring

Renal biopsies were evaluated by a single pathologist (REC) for glomerular and TI damage. Glomerular damage was evaluated with LM and TEM, and TI damage was evaluated with LM. A variation of the scoring system developed for the World Small Animal Veterinary Association Renal Standardization Project¹²⁸ was used to indicate the amount and severity of glomerular and TI damage (Tables A-5, A-6, and A-7). For TI damage, the final score consisted of an average of individual scores for each component of TI damage (interstitial fibrosis, tubular atrophy, degeneration/necrosis/regeneration, and interstitial chronic inflammation). Figures 2, 3, and 4 provide examples of the glomerular and TI damage scoring systems used in for evaluation of renal biopsies with LM and TEM.

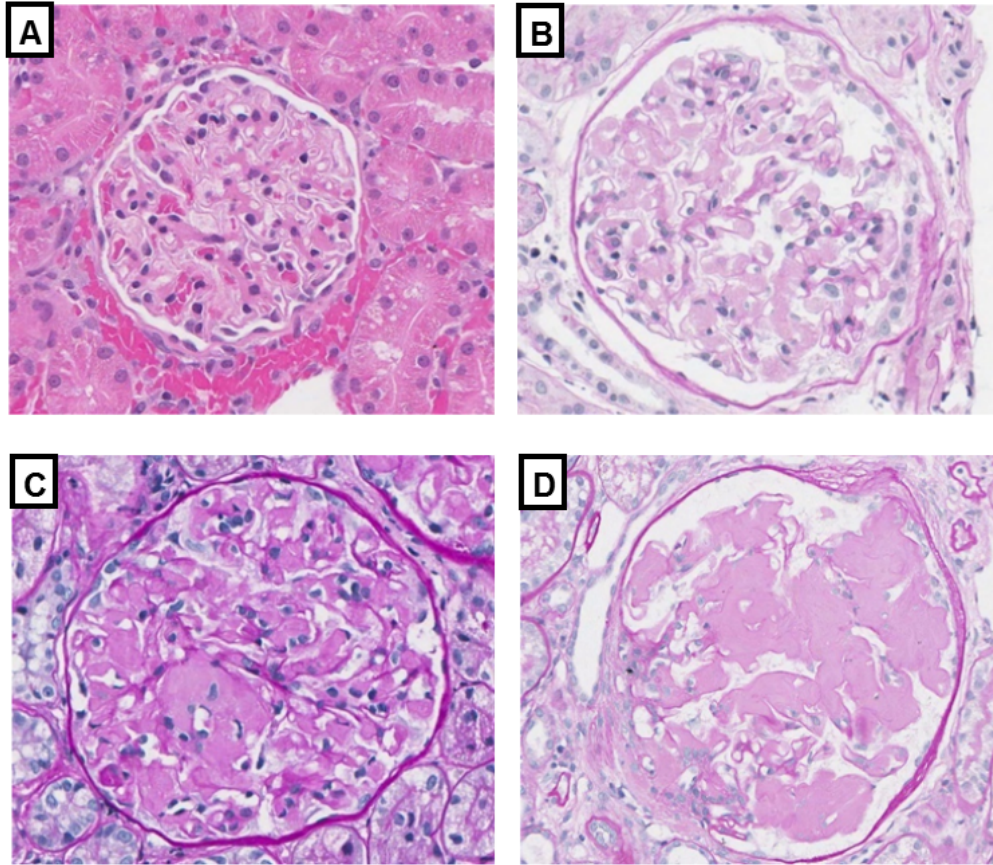


Figure 2. Light microscopic (LM) images from 1 dog with normal glomeruli and 3 dogs with varying severities of glomerular damage due to glomerular amyloidosis. A) Normal glomerulus; B) mild glomerular amyloidosis, LM glomerular biopsy score = 1; C) moderate glomerular amyloidosis, LM glomerular biopsy score = 2; D) severe glomerular amyloidosis, LM glomerular biopsy score = 3.

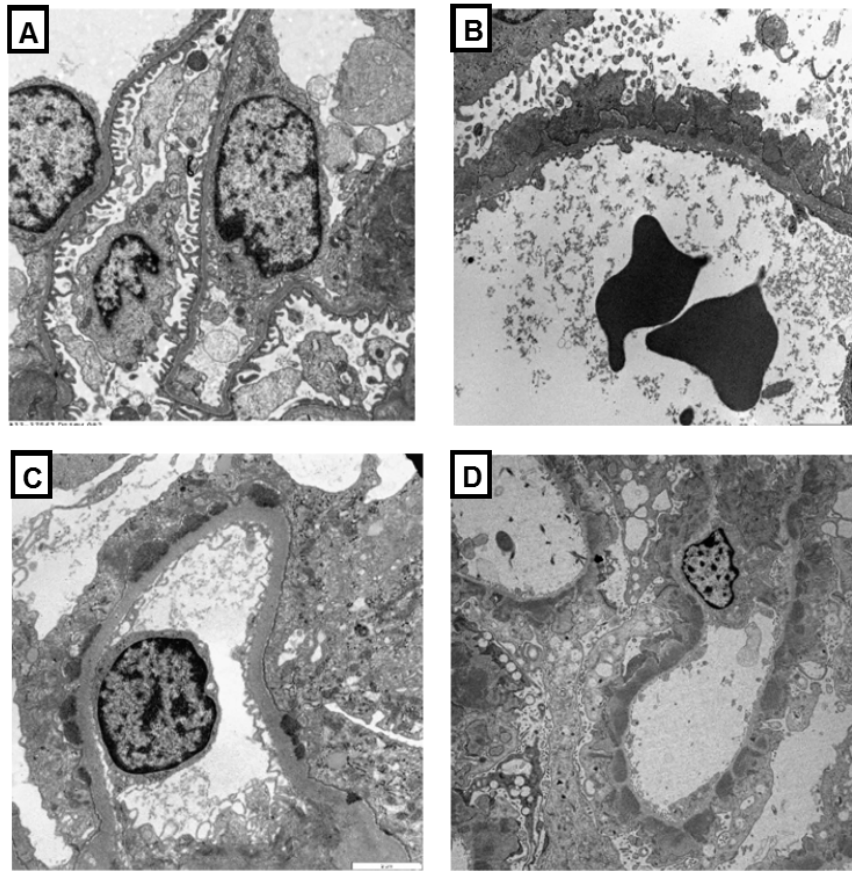


Figure 3. Transmission electron microscopic (TEM) images from 1 dog with normal glomeruli and 3 dogs with varying severities of membranous glomerulonephritis (MGN - a type of immune complex-mediated glomerulonephritis). A) Normal glomerulus; B) mild MGN, TEM glomerular biopsy score = 1; C) moderate MGN, TEM biopsy score = 2; D) severe MGN, TEM glomerular biopsy score = 3.

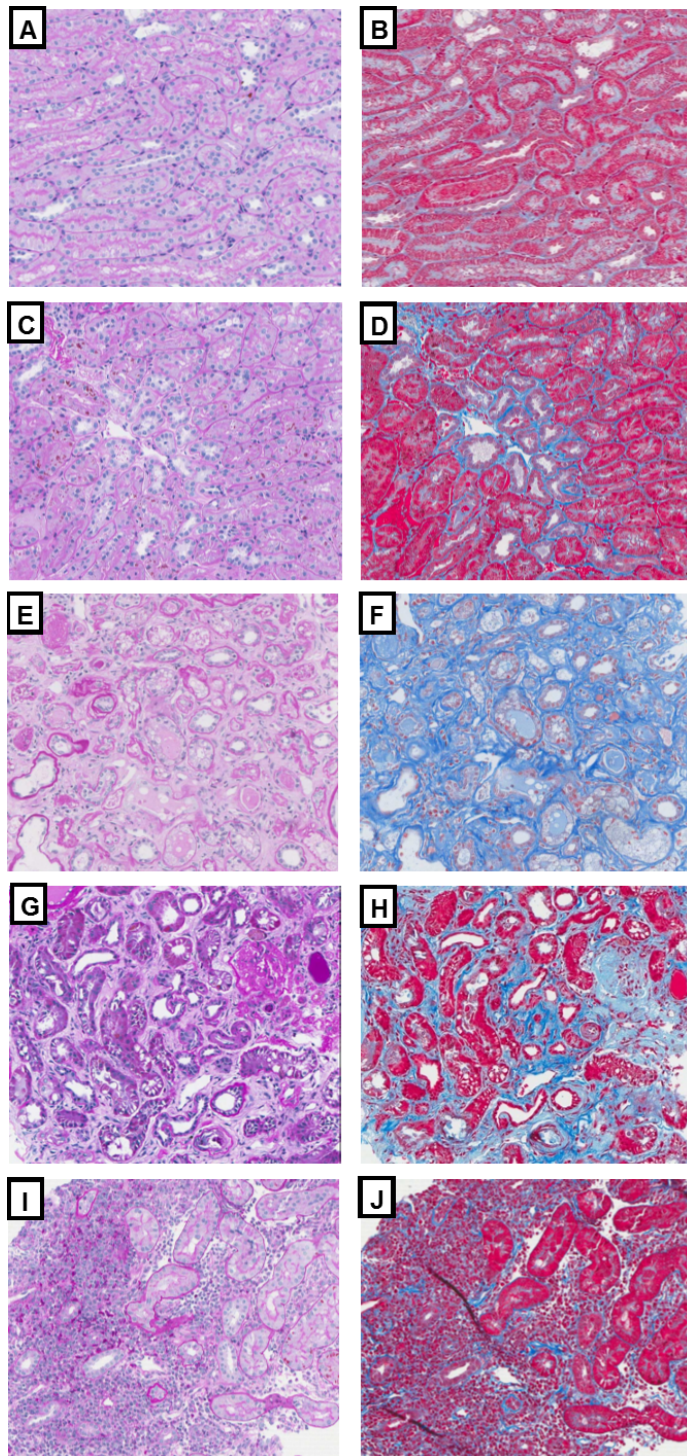


Figure 4. Light microscopic images from 1 dog with normal tubulointerstitium and 4 dogs with varying severities of tubulointerstitial (TI) damage. A (Periodic acid-Schiff (PAS) and B (trichrome): Interstitium and tubules are within normal limits; interstitial fibrosis score = 0, tubular atrophy score = 0, tubular degeneration score = 0, interstitial chronic inflammation score = 0; C (PAS) and D (trichrome): Mild to moderate multifocal interstitial fibrosis with tubular degeneration and atrophy; interstitial fibrosis score = 0.3, tubular atrophy score = 0.1, tubular degeneration score = 0.7, interstitial chronic inflammation score = 0.1; E (PAS) and F (trichrome): Moderate to severe interstitial fibrosis and tubular degeneration and atrophy; interstitial fibrosis score = 2.8, tubular atrophy score = 1.9, tubular degeneration score = 2.0, interstitial chronic inflammation score = 0.4; G (PAS) and H (trichrome): Severe diffuse interstitial fibrosis with tubular atrophy and degeneration; interstitial fibrosis score = 4.7, tubular atrophy score = 2.9, tubular degeneration score = 3.2, interstitial chronic inflammation score = 1.4; I (PAS) and J (trichrome): Severe diffuse chronic active lymphoplasmacytic interstitial nephritis with tubular degeneration, necrosis, and regeneration and extensive intraepithelial and intrahistiocytic gold brown pigment; interstitial fibrosis score = 3.4, tubular atrophy score = 0.1, tubular degeneration score = 4.1, interstitial chronic inflammation score = 4.3.

Assay Validation

Commercial assay kits for each biomarker (IgG,^a IgM,^b RBP,^c NGAL,^d NAG^e) were used. The IgG, RBP, and NAG assays were previously validated using canine urine.³⁷ Assay validation for IgM and NGAL can be found in the appendix.

Biomarkers

All urine and serum biomarkers were analyzed in duplicate. Freeze-thaw cycles were limited to ≤ 5 per sample. Conventional biomarkers (serum creatinine (sCr),^{f,g} urine protein:creatinine (UPC),^{f,g,h} and urine specific gravity (USG)ⁱ) and novel biomarkers (urine and serum IgG (uIgG and sIgG), IgM (uIgM and sIgM), RBP (uRBP and sRBP), and NGAL (uNGAL and sNGAL) and urine NAG (uNAG)) were measured in our laboratory. Standards were optimized according to the manufacturer for detection of RBP in canine samples. Urine biomarker concentrations were normalized to urine creatinine concentration (e.g., uIgG/c). Using the spot sample approach,¹²⁹ fractional excretion (FE) of IgG, IgM, RBP, and NGAL (IgG_FE, IgM_FE, RBP_FE, and NGAL_FE) were calculated using the formula:

$FE_{\text{analyte}} = (\text{Analyte}_{\text{urine}} / \text{Analyte}_{\text{serum}}) \times (\text{sCr} / \text{Creatinine}_{\text{urine}}) \times 100$. Cases were classified into

^a Dog IgG ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery, TX

^b Dog IgM ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery, TX

^c Human Retinol Binding Protein ELISA Kit, Immunology Consultants Laboratory, Inc., Newberg, OR

^d Dog NGAL ELISA Kit, Immunology Consultants Laboratory, Inc., Newberg, OR

^e N-acetyl- β -D-glucosaminidase (NAG) Assay, Diazyme Laboratories, Poway, CA

^f Creatinine LiquiColor Test (Endpoint), Stanbio Laboratory, Boerne, TX

^g Siron Clinical Chemistry Analyzer, Stanbio Laboratory, Boerne, TX

^h Protein, Micro LiquiColor Test (CSF and Urine), Stanbio Laboratory, Boerne, TX

ⁱ Rhino VET360 Veterinary Clinical Refractometer, Reichert Technologies, Depew, NY

CKD stages 1–4 based on the International Renal Interest Society (IRIS) guidelines^j, realizing that some cases could represent acute or acute on chronic disease and therefore not be in steady state.

Survival Data

The referring veterinarian or owner for each dog was contacted from 6 months to 6 years post-biopsy. If deceased, the following information was recorded: time to death post-biopsy, whether death was spontaneous or due to euthanasia, and cause of death (renal-related or otherwise).

Statistical Analysis

Biomarker Correlations

Simple linear regression on standardized continuous variables was used to estimate the correlation between biomarkers and also between each biomarker and biopsy damage scores to determine which biomarker correlated best with renal injury. Standardization was performed by subtracting the mean of the variable from an individual result and dividing by the standard deviation of that variable. Simple linear regression was also used to estimate the correlation between glomerular and TI damage scores. Correlation strength was defined as follows: weak: $r = 0.0-0.39$; moderate: $r = 0.4-0.69$; strong: $r = 0.7-1.0$. The Kolmogorov-Smirnov test was used to assess normality

^j <http://www.iris-kidney.com/guidelines/staging.shtml>

of the residuals, and data were natural log or square root transformed as necessary. Simple linear regression modeling for groups was used to determine significant differences in biomarkers between IRIS stages.

Disease Type Prediction

Logistic regression was used to determine presence of significant associations between biomarkers and specific types of kidney diseases, based on histopathological diagnosis, including: immune complex-mediated glomerulonephritis (ICGN), glomerulosclerosis, amyloidosis, other nephropathies, and tubular disease. “Other nephropathies” included juvenile nephropathies (e.g., maldevelopment) and nephropathies other than ICGN, glomerulosclerosis, amyloidosis, and primary tubular disease, such as cases with glomerular basement membrane and podocyte damage without immune complex deposition, cases with glomerular atrophy, and cases with primarily interstitial fibrosis. Receiver operator characteristic (ROC) analysis was performed to determine sensitivities and specificities for each biomarker with each disease type, and cutoff values for each biomarker were calculated based on that which maximized sensitivity and specificity. Sensitivities and specificities for disease types were also calculated for selected pairs of biomarkers. For each disease type, a dichotomous variable was created to be the response for the logistic model and ROC analysis (1 indicated the disease type of interest and 0 was otherwise).

Survival Analysis

For each biomarker and damage score, a survival model was fit, using a Cox semi-parametric model (accounting for the biomarker/damage score and age as covariates) to estimate median time to death due to renal disease post-biopsy using all follow-up data obtained ($n = 98$ dogs). Hazard ratios (HR) were used to describe the association of the biomarker and age with time to death. All data were also evaluated together in a multivariate Cox model to determine which combination of biomarkers and biopsy damage scores had the most significant association with time to death due to renal disease post-biopsy as described by HR. For the Cox models, TEM glomerular damage scores were re-categorized into “no damage to mild damage” (0 and 1) versus “moderate to severe damage” (2 and 3).

All statistical analyses were carried out using Stata version 13, setting $P < 0.05$.^k

Results

Dogs/Samples

Urine supernatant, serum, and kidney tissue from 203 dogs were initially analyzed. Of these, 130 (64%) urine samples had urinalyses performed on the submitted sample by the referring veterinarian or on a sample within 4 weeks of biopsy collection. Twenty-three dogs had evidence of an ongoing or recent bacteriuria, pyuria, or

^k Stata Corp. LP, College Station, TX

hematuria, and these cases were completely excluded leaving 180 cases for further analysis.

Of the remaining 180 cases, there were 80 (44.4%) spayed females, 57 (31.7%) neutered males, 25 (13.9%) intact males, and 18 (10.0%) intact females.

Numerous breeds were represented; the most common breeds were: Labrador Retrievers/Labrador Retriever-mixes: 19 (10.6%); Golden Retrievers/Golden Retriever-mixes: 9 (5.0%); Yorkshire Terrier/Yorkshire Terrier-mixes: 9 (5.0%); Miniature Schnauzers: 7 (3.9%); Doberman Pinschers: 6 (3.3%); and Rottweiler/Rottweiler-mixes: 5 (2.8%).

The age range was 2 months to 14 years old, with a median of 7 years old. Ten dogs (5.6%) were 0 to <1 year; 45 (25.0%) were 1 year to <5 years; 101 (56.1%) were 5 to <10 years; and 22 (12.2%) were ≥ 10 years. Two dogs were of an unknown age.

Follow-up information was collected for 98 (54%) dogs; information regarding time from biopsy to death and cause of death was collected for 62 dogs, 51 of which died or were euthanized due to renal-related causes. Median time to death due to renal disease post-biopsy (excluding submitted necropsy samples) was 179 days (range: 2–1,349 days).

Kidney disease was diagnosed based on persistent proteinuria in 87 dogs (48.3%), azotemia in 19 dogs (10.6%), and both proteinuria and azotemia in 74 dogs (41.1%). CKD was confirmed for 165 (91.7%) of dogs, while 3 dogs (1.7%) had concurrent CKD and AKI. Five dogs (2.8%) had AKI, and for 7 dogs (3.9%) chronicity of renal disease was unable to be determined.

Histopathologic Findings and Scores

Of 180 dogs included in the study, glomerular and TI damage were assessed in 176 dogs, whereas the remaining 4 did not have renal tissue available for evaluation. One hundred-fifty-one dogs had glomeruli available for evaluation by TEM, and the remaining 29 dogs did not have TEM performed for various reasons (e.g. LM evaluation was sufficient for diagnosis, a TEM sample was not submitted, or glomeruli were not present in the TEM sample). Table 1 demonstrates that this study cohort overall had worse glomerular damage than TI damage. Cases were divided into 5 disease categories with the following distribution: ICGN: 62 (34.4%); glomerulosclerosis: 47 (26.1%); amyloidosis: 18 (10.0%); other nephropathies: 32 (17.8%); and primary tubular disease: 15 (8.3%). Biopsies from 6 (3.3%) dogs were not assigned a disease category because the biopsied regions were either normal or insufficient to make a complete disease diagnosis; these cases were removed from the disease type prediction analysis. However, for 4 of these 6 cases, there was adequate tissue for either glomerular evaluation ($n = 2$), or TI evaluation ($n = 2$). Correlation between TI and LM glomerular damage scores was moderate ($r = 0.45$, $P < 0.001$), whereas there was no correlation between TI and TEM glomerular damage scores ($r = -0.03$).

Table 1. Percentage of cases in each category of glomerular damage biopsy scores based on LM and TEM and TI damage biopsy scores based on LM.¹²

	Score 0 - < 1	Score 1 - < 2	Score 2 - < 3	Score \geq 3
LM Glomerular Damage Score N = 176	8.5%	26.7%	40.3%	24.4%
TEM Glomerular Damage Score N = 151	2.0%	24.5%	43.7%	29.8%
TI Damage Score N = 176	58.5%	30.1%	10.8%	0.6%

LM, light microscopy; TEM, transmission electron microscopy; TI, tubulointerstitial.

Assay Validation

Analytical performance of the NGAL and IgM assays was acceptable (Table A-8).

Biomarker Findings

On average, dogs were mildly to moderately azotemic (107 dogs (59.4%) had $sCr \geq 1.4$ mg/dL) and moderately to markedly proteinuric (137 dogs (76.1%) had $UPC \geq 2.0$) (Table 2). Twenty-seven (15%) dogs had a UPC 0.5-2.0 and 16 (9%) had $UPC < 0.5$. Of these 43 dogs with $UPC < 2.0$, 23.3% had primary glomerular disease as determined by histopathology. Of the dogs with $UPC < 0.5$ (n=16), 1 dog (6.3%) had primary glomerular disease.

Biomarkers demonstrated a large range of values, and typically urine biomarkers were higher than have previously been reported in clinically healthy dogs.^{37,50,82,83,86,103} For 66.6% of the 180 cases included in the study, all biomarkers were measured; the remaining 33.4% of cases did not have a complete biomarker set. uIgG, uIgM, and uRBP were measured for 100% of the cases. sIgG, sIgM, and sRBP were measured for

all cases which had submitted serum samples (76.1% of cases). sNGAL and uNGAL were measured for 68.9% and 85% of cases, respectively. uNAG was measured for 94.4% of cases. When only cases that had a complete set of biomarker data were included in the statistical analyses, results of each analysis were similar to results when all cases (i.e., those with and without a complete biomarker set) were included (data not shown). While FE for most biomarkers was <100%, NGAL_FE ranged from 0-506%. With regard to IRIS stages, only RBP_FE and NGAL_FE demonstrated significantly progressive increases with higher IRIS stages, although all novel biomarkers except uIgG/c and uNAG/c tended to increase with higher stages of disease (Table A-9).

Table 2. Median (range) of biomarker values in dogs with naturally occurring chronic kidney disease. For biomarkers that were significantly associated with specific categories of disease according to logistic regression, optimal cutoff values and corresponding sensitivities and specificities, as determined by receiver operator characteristic (ROC) analysis, are displayed. ¹²												
	n	All Dogs Median (Range)	n	ICGN Median (Range) Cutoff (Se%, Sp%)	n	Glomerulosclerosis Median (Range) Cutoff (Se%, Sp%)	n	Amyloidosis Median (Range) Cutoff (Se%, Sp%)	n	Other Nephropathies Median (Range) Cutoff (Se%, Sp%)	n	Tubular Disease Median (Range) Cutoff (Se%, Sp%)
sCr (mg/dL)	179	1.5 (0.3 - 21.8)	61	1.5 (0.3 - 9.3)	47	1.4 (0.3 - 12.0)	18	1.1 (0.5 - 4.9)	32	1.7 (0.6 - 21.8)* >2.5 (37.5, 78.2)	15	2.1 (0.5 - 13.0)* >3.2 (46.7, 87.2)
USG	180	1.017 (1.003 - 1.048)	62	1.019 (1.005 - 1.046)** >1.014 (80.6, 47.5)	47	1.017 (1.003 - 1.048)	18	1.013 (1.003 - 1.027)* <1.013 (50.0, 73.5)	32	1.014 (1.005 - 1.047)	15	1.013 (1.005 - 1.026)* <1.014 (60.0, 69.1)
UPC Ratio	180	5.0 (0.0 - 36.8)	62	8.6 (0.4 - 36.8)** >2.5 (95.2, 40.7)	47	5.2 (0.5 - 31.6)	18	11.0 (1.7 - 21.3)	32	1.5 (0.0 - 14.3)** <2.5 (71.9, 81.8)	15	0.5 (0.0 - 6.0)** <2.0 (86.7, 81.2)
IgG												
uIgG/c (µg/mg)	180	554.0 (0.6 - 37,649.0)	62	1,212 (27 - 37,649)** >729.0 (62.9, 72.9)	47	562.5 (48.7 – 7,070.3)	18	727.0 (128.9 - 6,589.0)	32	104.1 (0.6 - 1,938.5)** <374.7 (87.5, 71.6)	15	37.3 (0.7 - 489.3)** <211.0 (86.7, 80.0)
sIgG (µg/ml)	137	11,320 (3,199 – 41,798)	48	11,288 (3,199 – 29,686)	36	11,280 (6,482 – 41,798)	12	9,251 (3,669 – 20,185)	27	10,670 (3,742 – 26,785)	10	14,933 (9,098 – 31,108)
IgG_FE%	137	0.1 (0.0 - 8.6)	48	0.1 (0.0 - 8.6)	36	0.1 (0.0 - 1.4)	12	0.2 (0.0 - 3.2)	27	0.0 (0.0 - 4.7)	10	0.0 (0.0 - 0.2)
IgM												
uIgM/c (µg/mg)	180	6.4 (0.3 - 557.0)	62	14.6 (2.1 - 557.0)** >8.0 (74.2, 75.4)	47	5.6 (0.5 - 26.9)* <13.5 (87.2, 32.3)	18	5.7 (3.1 - 24.3)	32	3.1 (0.4 - 20.2)** <5.6 (75.0, 60.1)	15	2.0 (0.3 - 28.3)* <3.5 (73.3, 74.5)
sIgM (µg/ml)	137	2,864 (867 – 20,070)	48	3,130 (867 – 20,070)* >4433.0 (31.3, 89.9)	36	2,468 (1,033 – 7,512)	12	3,057 (1,051 – 7,717)	27	2,774 (1,185 – 3,906)	10	3,503 (2,056 – 10,921)
IgM_FE%	137	0.003 (0.000 - 0.172)	48	0.008 (0.000 - 0.145)* >0.005 (62.5, 76.4)	36	0.002 (0.000 - 0.022)* <0.005 (83.3, 45.5)	12	0.002 (0.001 - 0.019)	27	0.002 (0.0 00 - 0.172)	10	0.001 (0.000 - 0.033)
RBP												
uRBP/c (µg/mg)	180	8.7 (0.0 - 1,013.4)	62	9.3 (0.1 - 243.8)	47	10.0 (0.0 - 1,013.4)	18	2.1 (0.0 - 78.4)	32	4.0 (0.0 - 402.9)	15	1.6 (0.0 - 306.1)
sRBP (µg/mL)	137	105.5 (22.9 - 414.2)	48	90.1 (22.9 - 280.5)* <112.8 (70.8, 55.1)	36	139.6 (30.9 - 258.2)** >112.2 (75.0, 64.4)	12	80.9 (44.2 - 126.0)	27	109.2 (26.7 - 414 .2)	10	96.2 (39.3 - 160.1)
RBP_FE%	137	0.1 (0.0 - 24.9)	48	0.2 (0.0 - 18.0)	36	0.1 (0.0 - 2.7)	12	0.1 (0.0 - 3.2)	27	0.0 (0.0 - 14.3)	10	0.0 (0.0 - 24.9)* >1.5 (30.0, 89.8)
NGAL												
uNGAL (ng/mg)	153	93.6 (0.0 - 1,533.4)	53	107.5 (8.2 - 1,533.4)	41	102.6 (9.7 - 842.1)	13	92.2 (13.1 - 321.7)	29	63.1 (0.0 - 660.6)	11	43.5 (4.2 - 496.9)
sNGAL (ng/mL)	124	12.1 (2.4 - 149.1)	44	14.0 (2.6 - 65.7)	34	13.1 (3.3 - 149.1)	10	11.0 (4.8 - 23.4)	24	8.9 (2.4 - 106.8)	7	8.3 (2.8 - 24.7)
NGAL_FE%	124	10.8 (0.0 - 505.9)	44	11.7 (0.5 - 82.8)	34	10.1 (1.9 - 86.1)	10	13.3 (2.2 - 40.4)	24	7.5 (0.0 - 505.9)	7	3.6 (0.2 - 26.8)
uNAG (U/g)	170	13.5 (0.4 - 427.7)	57	26.5 (3.0 - 427.7)** >23.8 (56.1, 85.0)	46	12.7 (2.0 - 76.7)	18	20.4 (3.3 - 67.2)	30	7.4 (0.6 - 37.7)** <9.4 (63.3, 71.4)	13	2.8 (0.4 - 10.3)** <6.9 (92.3, 79.6)

Biomarker is significantly associated with type of kidney disease according to logistic regression: *P<0.05, **P<0.01; Outcome for ROC analysis in determining sensitivity and specificity was presence of a particular disease. Cutoff values were determined by those which maximize the area under the ROC curve, and greater or less than the cutoff value was determined by the increased probability of having the disease when above or below the cutoff value. ICGN, immune complex-mediated glomerulonephritis; Se%, sensitivity; Sp%, specificity; sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; sIgG, serum immunoglobulin G; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; sIgM, serum immunoglobulin M; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

Biomarker Correlations

Of 45 combinations of urine protein biomarkers (normalized urine concentration or FE), 82.2% showed moderate to strong correlations with each other and with UPC (Table A-10). In contrast, serum protein biomarker concentrations (sRBP and sNGAL) generally demonstrated weak correlations with other biomarkers, and the highest correlation was observed with the urine concentration of the same biomarker (e.g., sRBP with uRBP/c). sCr correlated only weakly to moderately with FE of the biomarkers, with the strongest (but still moderate) correlation for sCr being with RBP_FE.

Glomerular damage based on LM correlated best (albeit moderately) with FE of high molecular weight (i.e., “glomerular”) biomarkers (IgM_FE: $r = 0.58$; IgG_FE: $r = 0.56$), and both IgM_FE and IgG_FE had stronger correlations with glomerular damage than did UPC ($r = 0.45$) (Figure 5). The remaining urine protein biomarkers (urine concentrations and FEs) correlated less strongly with glomerular damage (range: $r = 0.32$ - 0.47). Correlations of many biomarkers with glomerular damage were stronger when based on TEM compared with LM (Figure 6).

For TI damage, sCr had the strongest correlation ($r = 0.7$, Figure 7). RBP was the only biomarker where all measurements (urine and serum) significantly correlated with TI damage, with RBP_FE demonstrating the strongest, albeit moderate, correlation ($r = 0.58$). The only other significant correlations with TI damage were FEs of the other biomarkers as well as USG, which demonstrated a weak, negative correlation.

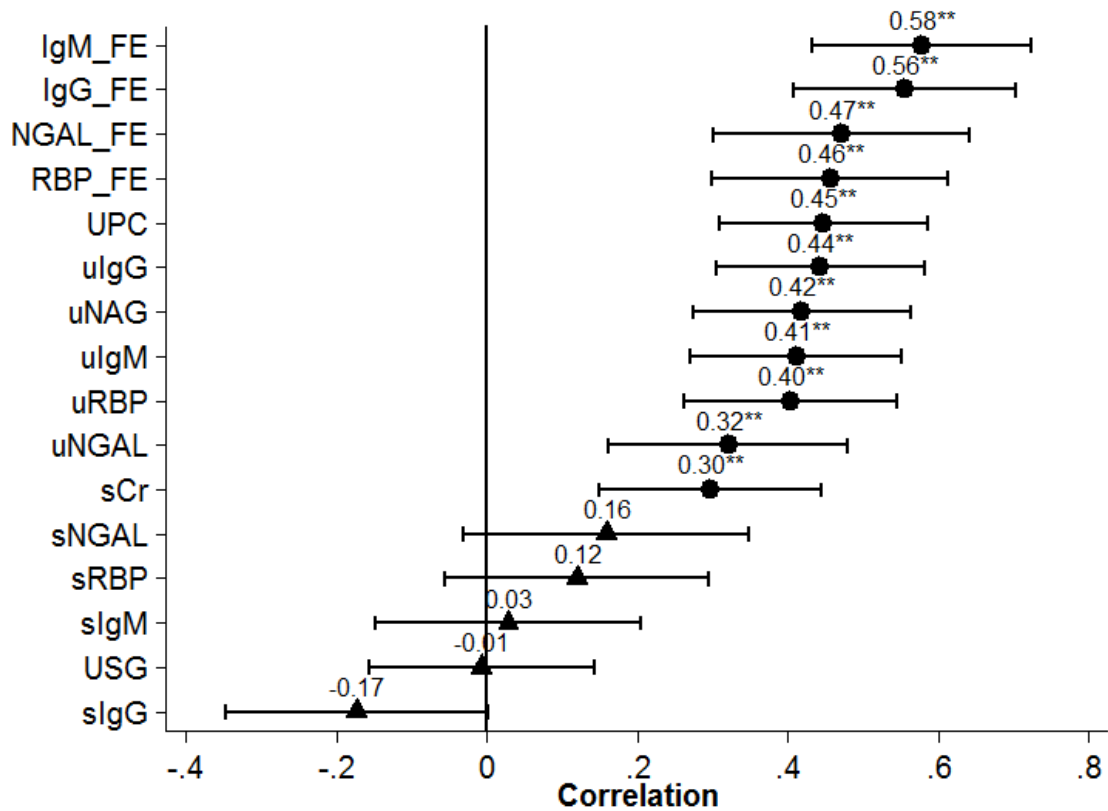


Figure 5. Correlations of biomarkers with glomerular damage based on light microscopy, with 95% confidence intervals. Circles represent a statistically significant correlation and triangles represent no significant correlation. $**P < 0.01$; $n = 176$. sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; ulgG/c, urine immunoglobulin G/urine creatinine; slgG, serum immunoglobulin G; IgG_FE, fractional excretion of immunoglobulin G; ulgM/c, urine immunoglobulin M/urine creatinine; slgM, serum immunoglobulin M; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl- β -D-glucosaminidase/urine creatinine.¹²

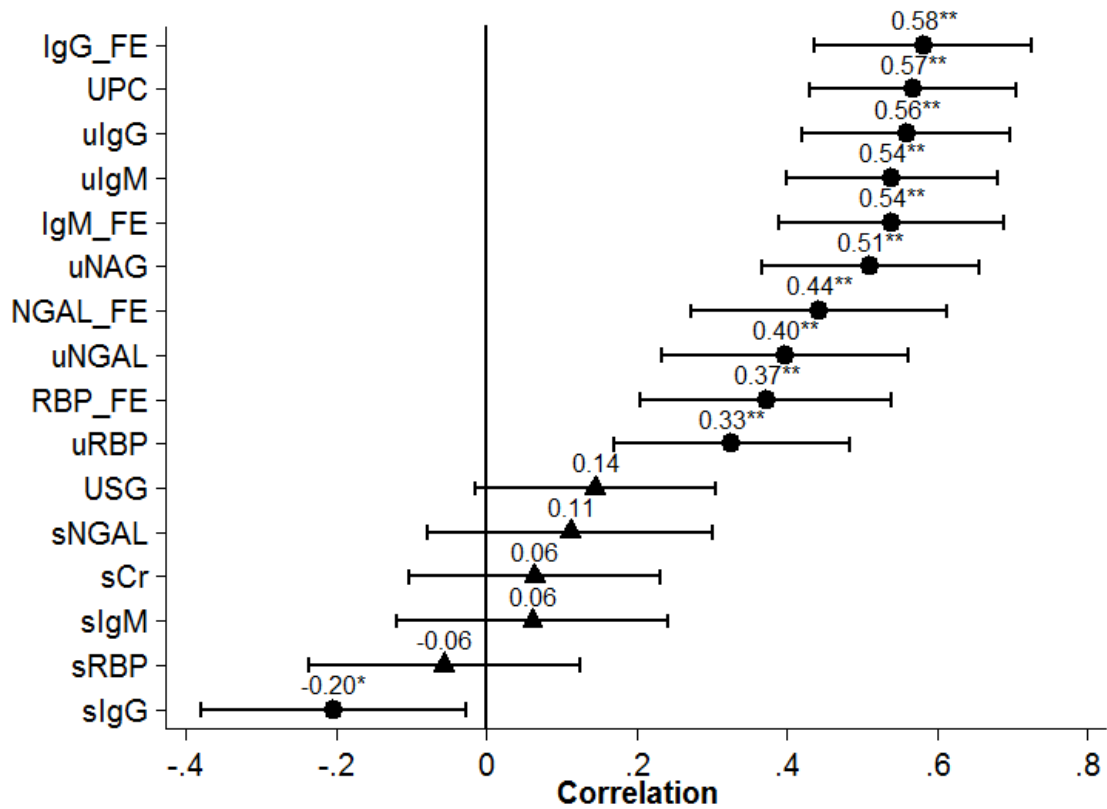


Figure 6. Correlations of biomarkers with glomerular damage based on transmission electron microscopy with 95% confidence intervals. Circles represent a statistically significant correlation and triangles represent no significant correlation. ** $P < 0.01$; $n = 151$. sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; sIgG, serum immunoglobulin G; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; sIgM, serum immunoglobulin M; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl- β -D-glucosaminidase/urine creatinine.¹²

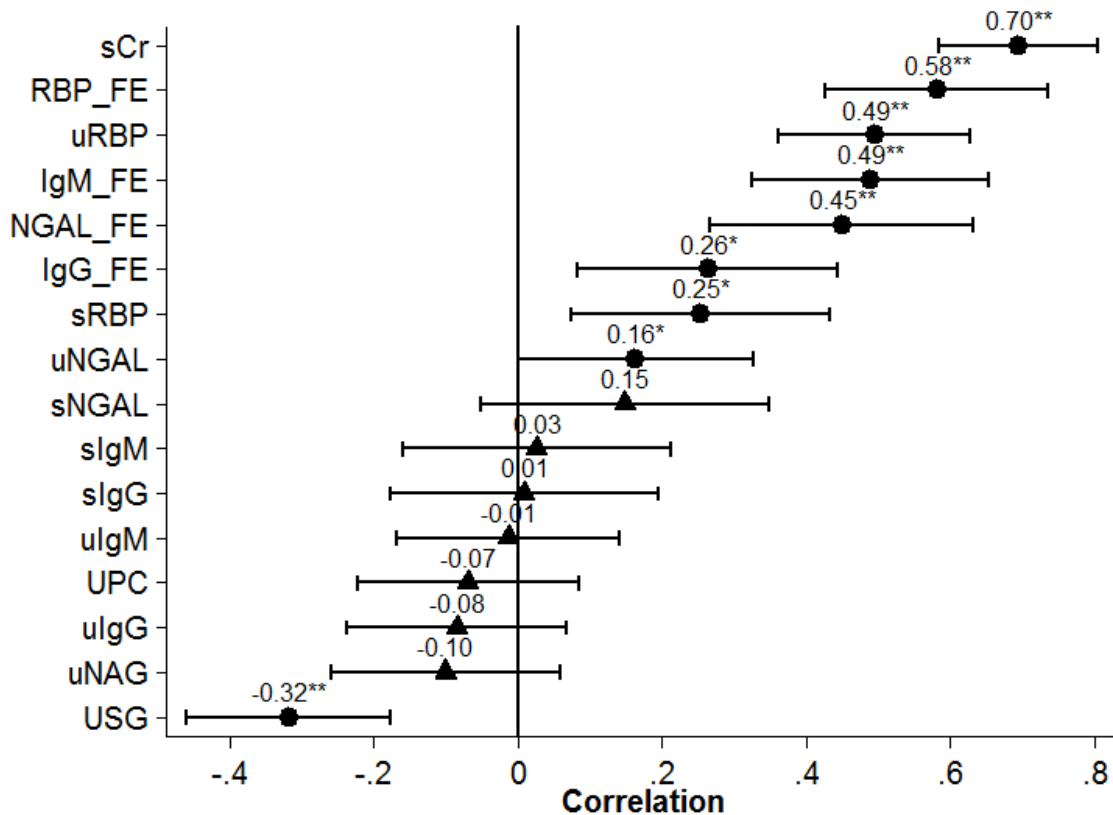


Figure 7. Correlations of biomarkers with tubulointerstitial damage based on light microscopy with 95% confidence intervals. Circles represent a statistically significant correlation and triangles represent no significant correlation. * $P < 0.05$; ** $P < 0.01$; $n = 176$. sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; sIgG, serum immunoglobulin G; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; sIgM, serum immunoglobulin M; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl- β -D-glucosaminidase/urine creatinine.¹²

Disease Type Prediction

We used logistic regression to determine if any of the biomarkers was significantly associated with each diagnostic category (Table 2). ROC analysis and sensitivity and specificity calculations for each disease type were also performed for each biomarker. Individually, increased uIgM/c, uIgG/c, uNAG/c, and UPC were significantly associated with ICGN based on logistic regression, and these demonstrated the highest sensitivities and specificities for ICGN. A ROC analysis of pairs of biomarkers revealed uIgM/c and uNAG/c to be the only combination of biomarkers significantly associated with ICGN. This combination, using cutoff values of uIgM/c >7.3 µg/mg and uNAG/c >7.0 U/g, had a sensitivity and specificity for detection of ICGN of 75% and 78%, respectively, which was similar to that of uIgM/c alone. Individually, low UPC, uIgG/c, and uNAG/c were most significantly associated with primary tubular disease; however, ROC analysis did not reveal a combination of biomarkers that was significantly associated with primary tubular disease.

Survival Analysis

In this cohort of dogs with primarily proteinuric CKD, increases in sCr, IgM_FE, uRBP/c, RBP_FE, NGAL_FE, and IgG_FE, as well as TI, LM and TEM glomerular damage scores were all significantly associated with shortened time to death due to renal disease according to Cox survival models including age as a covariate (Table 3). For example, an increase in sCr of 1 mg/dL resulted in an increased hazard of death of 40%, while an increase of 0.01% for IgM_FE resulted in an increased hazard of death of 45%.

An increase in TI damage score or LM glomerular damage score of 1 point (e.g., 0 to 1 or 1 to 2) resulted in increased hazards of death of 160% and 60%, respectively, while an increase in TEM glomerular damage score from 0/1 to 2/3 resulted in an increased hazard of death of 158%. Age also had a significant association with time to death when combined in the survival models for IgM_FE, RBP_FE, and IgG_FE (i.e., a 1-year increase in age increased the hazard of death associated with these biomarkers). Age did not have a significant association with time to death when combined in survival models for sCr, uRBP/c, NGAL_FE, TI damage score, and LM or TEM glomerular damage scores.

To determine which combination of biomarkers and damage scores was significantly associated with time to death due to renal disease in this cohort of dogs, all biomarkers were evaluated together in a multivariate Cox survival model. Notably, sCr, IgM_FE, and TEM glomerular damage scores were the only parameters that were significantly associated with time to death due to renal disease post-biopsy (Table 4). Survival graphs for these three variables demonstrate the probability of survival for a dog of median age (7 years) based on different starting levels of each biomarker or damage score (Figure 8). Survival graphs also demonstrate the probability of survival for dogs with varying combinations of sCr and IgM_FE values based on a TEM glomerular damage score of either 0/1 or 2/3 (Figure A-1).

Table 3. Association of biomarker/damage score and age with time to death due to renal disease in dogs from multivariate Cox survival models. The unit increase for each biomarker/damage score and age is depicted in parentheses in the first column. Each row depicts a separate survival model that includes 2 covariables (biomarker or damage score and age).¹²

		HR for Biomarker/Damage Score		HR for Age	
		HR (95% CI)	P value	HR (95% CI)	P value
Biomarker + Age (1 year)	N				
sCr (1 mg/dL)	82	1.40 (1.26-1.56)	<0.001	1.10 (0.99-1.22)	0.072
IgM FE (0.01%)	66	1.45 (1.25-1.69)	<0.001	1.15 (1.00-1.32)	0.047
uRBP/c (10 µg/mg)	83	1.07 (1.03-1.10)	<0.001	1.07 (0.98-1.18)	0.14
RBP FE (1%)	66	1.17 (1.07-1.28)	0.001	1.16 (1.01-1.33)	0.034
NGAL FE (25%)	59	1.23 (1.07-1.41)	0.003	1.09 (0.95-1.25)	0.20
IgG FE (1%)	66	1.47 (1.11-1.95)	0.007	1.15 (1.00-1.31)	0.044
Damage Score + Age (1 year)					
TI Damage Score (1 Score Point)	82	2.60 (1.59-4.24)	<0.001	1.07 (0.97-1.17)	0.17
LM Glomerular Damage Score (1 Score Point)	82	1.60 (1.12-2.29)	0.009	1.08 (0.98-1.20)	0.13
TEM Glomerular Damage Score (0/1 vs. 2/3)	72	2.58 (1.03-6.44)	0.042	1.05 (0.94-1.18)	0.37

HR, hazard ratio; CI, confidence interval. Abbreviation explanations in Table 1 and 2 legends.

Table 4. Association of biomarkers and TEM glomerular damage score with time to death due to renal disease in dogs from a multivariate Cox survival model (n=84). The unit increase for each biomarker/damage score is depicted in parentheses in the first column.¹²

Biomarker/Damage Score	HR (95% CI)	P value
sCr (1 mg/dL)	1.40 (1.15-1.70)	0.001
IgM FE (0.01%)	1.28 (1.07-1.55)	0.008
TEM Glomerular Damage Score (0/1 vs. 2/3)	4.80 (1.32-17.50)	0.017

Abbreviation explanations in Table 1, 2, and 3 legends.

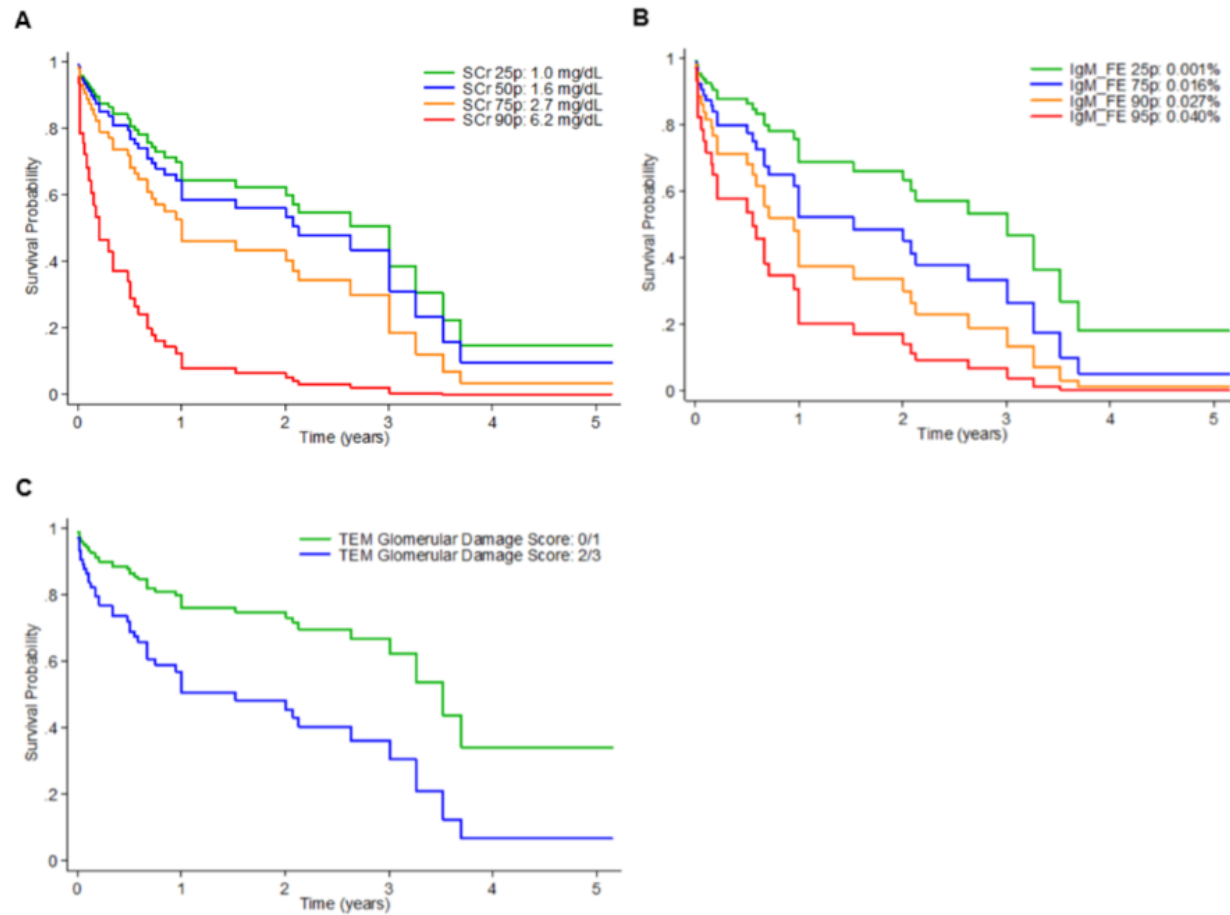


Figure 8. Probability of survival by biomarker/damage score for a dog at median age (7 years) at different starting values of biomarkers/damage scores for A) sCr (n=83); B) IgM_FE (n=67); C) TEM Glomerular Damage Score (n=73). 25p: 25th percentile; 50p: 50th percentile; 75p: 75th percentile; 90p: 90th percentile; 95p: 95th percentile. Additional abbreviation explanations in Table 1 and 2 legends.¹²

Discussion

This study evaluated 5 novel biomarkers in 180 dogs with naturally occurring kidney disease, primarily proteinuric CKD typically due to primary glomerular disease. Several of these urine biomarkers and their FEs correlated with pathologic severity of glomerular damage, TI damage, or both. Increased uIgM/c and uNAG/c were most significantly associated with ICGN. Furthermore, increased sCr, IgM_FE, uRBP/c, RBP_FE, NGAL_FE, and IgG_FE, as well as TI damage scores and LM and TEM glomerular damage scores were associated with reduced survival when variables were modeled separately. However, in a multivariate model, only sCr, IgM_FE, and TEM glomerular damage scores were associated with time to death due to renal-related causes. Our findings support that conventional markers of kidney disease (sCr, UPC) correlate with kidney damage either similarly to or better than the novel biomarkers evaluated in this study. However, novel biomarkers can provide useful additional information to support the presence of glomerular or tubular damage, to help distinguish between disease categories, and to inform prognosis.

This study did not reveal superiority of any novel biomarker to sCr with regard to TI damage or survival. However, sCr is often not optimally interpreted, with diagnosis of azotemia based on exceeding a reference interval rather than what is “normal” or baseline for a particular animal. This is especially problematic for small breed dogs and animals with concurrent muscle wasting, the latter of which is a common finding in CKD and will complicate monitoring for disease progression.¹¹⁷ Therefore, having additional non-invasive markers of TI damage and dysfunction would be useful to

confirm sCr interpretations. Additionally, even though sCr demonstrated the best correlation with TI damage, the majority (59%) of dogs evaluated in this study were already at least mildly azotemic at the time of biopsy, and it is possible that evaluation of urine biomarkers might help identify TI damage earlier than sCr.

IgG_FE and IgM_FE correlated slightly stronger than UPC with severity of glomerular damage when evaluated with LM; however, when glomerular damage was assessed with TEM, IgG_FE, UPC, and uIgG/c provided the best indications of ultrastructural glomerular damage. Thus, UPC is likely an effective conventional marker of ultrastructural glomerular damage. Of interest is the use of uIgM/c, uIgG/c, and uNAG/c, particularly the combination of uIgM/c and uNAG/c, for the identification of ICGN (discussed further below). Increased IgM_FE was also associated with a significantly increased hazard of death. This is similar to studies in humans, where increased uIgM/c excretion in diabetic glomerulonephropathy was shown to be associated with increased risk of renal failure and death.^{130,131} Because IgM is a large protein (~900 kDa), its presence in the urine might reflect more severe and possibly irreversible damage to the glomerular filtration barrier. In contrast to a previous study in dogs,¹²⁶ UPC was barely associated with survival ($P = 0.03$; $HR = 1.04$). This could be because this previous study included dogs with sCr ranging from 2.0 – 8.0 mg/dL,¹²⁶ whereas many of the dogs in the present study were not azotemic, and some had reversible glomerular injury. Treatment to reduce proteinuria both before and after renal biopsy might have also influenced results in our study.

The tubular markers sCr, RBP_FE, uRBP/c, and NGAL_FE typically correlated more strongly with TI than glomerular damage. However, all but sCr also correlated moderately well with glomerular damage. Similarly, IgM_FE correlated moderately with TI damage, although a stronger correlation was observed with glomerular damage. This is similar to a study of urine biomarkers in dogs with X-linked hereditary nephropathy, where sCr, uRBP/c, uNGAL/c, uNAG/c, and uIgG/c all correlated moderately to strongly with both glomerular and tubular lesions.³⁷ One possible explanation is that concurrent glomerular and tubular damage commonly occurs in dogs with CKD, and certainly, damage to one compartment will affect the other.

The use of TEM in our study identified biomarkers that were better for differentiating glomerular from tubular disease, including UPC, uIgG/c, uIgM/c, uNAG/c, and IgG_FE. Notably, most “glomerular” biomarkers had a stronger correlation with the TEM assessment of glomerular damage compared to LM evaluation, suggesting that TEM is better for determining the severity of glomerular filtration barrier damage. Furthermore, glomerular damage based on TEM, but not LM, was significantly associated with survival time in a multivariate survival model and therefore may be more predictive of prognosis. However, both LM and TEM are needed for the comprehensive assessment of kidney biopsies. LM allows for evaluation of many glomeruli, which is particularly important for identification of scattered sclerotic or obsolescent glomeruli. TEM provides a more detailed structural view of the glomerulus, particularly the glomerular filtration barrier, but is not routinely performed outside a specialized biopsy

service. In addition, it is usually performed on small tissue samples in which glomeruli may be absent.

Interestingly, uNAG/c correlated as strongly with glomerular damage as the glomerular markers but did not significantly correlate with TI damage. NAG is a tubular lysosomal enzyme recognized as a marker of tubular injury, wherein tubular damage causes release of NAG and subsequent elevation of enzyme activity in urine.^{47,132} NAG, which is approximately the size of IgG, does not pass through a normal glomerular filtration barrier, and the upper reference limit for uNAG/c in healthy dogs (3.63 U/g)¹⁰³ is well below the mean in our study. Previous studies have shown increased uNAG/c in dogs with CKD, presumed to be due to tubular damage or increased lysosomal turnover secondary to proteinuria.^{37,50,105} While it is still possible that NAG leakage is occurring without histologic evidence of tubular damage, the strong correlation with glomerular damage and lack of correlation with TI damage in our study supports the possibility that NAG can pass through an injured glomerular filtration barrier. Therefore, while uNAG/c has been used to detect tubular damage in cases of acute kidney injury, it might also be useful to detect glomerular damage in chronic proteinuric nephropathies.

Another unexpected finding was the similar correlation of IgM_FE with both TI and glomerular damage. This is particularly intriguing given that uIgM/c did not correlate with TI damage. Fractional excretions of RBP, NGAL, and IgG also correlated more strongly with TI damage than their urine concentrations. This suggests that determination of FE could be more valuable than urine concentration of these markers

for assessment of tubular damage, possibly due to the decreased ability of damaged tubular epithelial cells to reabsorb these proteins.

Maximum FEs observed for most biomarkers were <100%, except for NGAL_FE which reached 506%. This could indicate a large amount of secretion or loss of NGAL from damaged tubular cells. Alternatively, pyuria might have been present in samples that did not have a concurrent sediment examination despite recent results indicating inactive urine sediments. However, NGAL_FE in samples with known pyuria only reached up to 227% (data not shown).

An intriguing aspect of our study is the possibility that certain biomarkers might be able to predict specific disease types. In particular, markedly increased uIgM/c, uNAG/c, uIgG/c, and UPC were significantly associated with ICGN, which may be due to immune deposits creating large “holes” within the glomerular filtration barrier. A combination of uIgM/c and uNAG/c had a sensitivity and specificity for ICGN of 75% and 78%, respectively. While these values are not considerably high, they demonstrate promise in our ability to detect ICGN without a renal biopsy. However, further studies with larger populations of dogs are needed. While low UPC, uIgG/c, and uNAG/c were significantly associated with primary tubular disease on individual analysis, bivariate analysis did not reveal a combination of biomarkers with improved sensitivity or specificity over the individual analyses. It is possible that this might be due to an insufficient number of cases with primary tubular disease.

It was expected that TI damage score and sCr would be significantly associated with prognosis since TI damage is most closely associated with clinical parameters

(glomerular filtration rate and sCr).¹³³ However, many parameters (uRBP/c, RBP_FE, IgM_FE, IgG_FE, and NGAL_FE, and LM and TEM glomerular damage scores) were also significantly associated with survival time. Since uRBP/c was shown to increase earlier than sCr,³⁷ increases in urinary biomarkers might provide an earlier indication of prognosis in dogs with renal disease. In a multivariate survival model, sCr was the most informative biomarker. However, IgM_FE and TEM glomerular damage scores were also significantly associated with survival time, and IgM_FE was more strongly associated than TEM findings. This suggests that the combination of sCr and IgM_FE might better predict prognosis than biopsy findings, although renal biopsy remains the gold standard for diagnosis and guide to therapy. Even very small changes in IgM_FE predicted significant changes in prognosis. This could be because IgM is a very large protein, likely requiring more severe damage to the glomerulus in order to pass into the urine filtrate. TI damage score, on the other hand, was not significantly associated with survival time in the multivariate analysis, possibly because many of the cases in this study were biopsied for suspected nonazotemic glomerular disease and therefore had low TI damage scores. Finally, increasing age was irrelevant to survival time once biomarker values and damage scores were known. Given that these survival predictions are based primarily on a population of dogs with proteinuric CKD, typically due to glomerular disease, these biomarkers may not hold the same prognostic value in dogs with other etiologies of kidney disease.

Because most of the dogs in this study had proteinuric CKD, there was a bias for dogs with glomerular disease, which is a limitation of this study. Even so, 43 dogs (24%)

had UPC < 2, which is not typically considered indicative of primary glomerular disease, and 16 of these dogs (9% of the entire cohort) had a UPC < 0.5. Of the 43 dogs with UPC < 2, 23% had histologic evidence of primary glomerular disease. Of those with UPC < 0.5 (n=16), only 1 dog (6%) had primary glomerular disease. The distribution of the inciting cause of CKD in the general canine population is currently unknown, as it has not been comprehensively studied using clinicopathologic data, LM, and TEM; however, glomerular damage and proteinuria is common in dogs with CKD. While this study cohort does not completely represent the general population of dogs with CKD, a wide variety of naturally occurring kidney diseases were included.

A second limitation of the study is that not all urine samples had a corresponding urinalysis. The majority (64%) of the samples had a corresponding urinalysis performed either on the urine sample submitted with the biopsy or within 4 weeks of the biopsy. Most samples were collected by internists at referral centers who performed a complete medical evaluation, minimizing the likelihood that significant sediment abnormalities were present when urinalysis results were not provided. However, studies have demonstrated that urinary tract infections and hematuria might alter biomarker levels,⁸¹ and presence of infection could potentially increase systemic immunoglobulins. Therefore, all cases with known or suspected pyuria, bacteriuria, and/or marked hematuria were excluded from analyses to avoid interpreting increases in biomarkers that might be due to infection or hematuria.

Additional limitations include the unknown stability of the urine biomarkers, the variable time between collection and processing (although typically just one day), and

the variability in length of sample storage prior to biomarker determination. The use of spot samples for calculation of FE, while an accepted method, is considered less accurate than the clearance approach for calculation of FE.¹²⁹ However, the spot sample approach is more feasible in clinical practice. Additionally, not all biomarkers were measured in each dog; however, this was unlikely to have skewed the data as results were similar even if analysis was performed only for those cases with a complete biomarker set.

In conclusion, use of conventional biomarkers that are currently available for the diagnosis and monitoring of kidney disease, particularly sCr and UPC, are reasonable for assessment of kidney disease if used appropriately. A number of novel biomarkers are useful to detect glomerular or TI damage and potentially predict specific disease types and survival in dogs with naturally occurring CKD. Additionally, analysis of quantitative pathologic biopsy scores, as based on the recently published World Small Animal Veterinary Association manuscript¹²⁸ can aid in prognostication in dogs with CKD. More studies are needed using a larger cohort of dogs to determine if specific biomarkers such as uIgM/c and uNAG/c can help non-invasively diagnose ICGN in dogs. Furthermore, although uNAG/c has been reported as a marker of TI damage, it might be better suited as a marker of glomerular damage in dogs with proteinuric nephropathies.

CHAPTER III

URINARY IMMUNOGLOBULIN M AS A NOVEL BIOMARKER OF IMMUNE
COMPLEX MEDIATED GLOMERULONEPHRITIS IN DOGS

Introduction

Glomerular disease has been reported as a common cause of chronic kidney disease (CKD), found in approximately 50% to 70% of dogs with naturally occurring kidney diseases.^{11,134-137} Glomerular disease in dogs is often believed to be immune-mediated (immune complex mediated glomerulonephritis (ICGN)), with nearly 50% of cases of dogs with suspected glomerular disease having evidence of immune complexes present in the glomerulus.^{10,11,138-141} Canine ICGN is generally not a true auto-immune disease, in which antibodies are produced against glomerular antigens. Rather, the term “immune-mediated” is used to denote the presence of circulating immune complexes being deposited in the glomerulus or forming in the glomerulus secondary to deposited or entrapped foreign antigen.^{141,142} While infectious diseases and inflammatory diseases can incite glomerular deposition of immune complexes, the source can also be idiopathic.¹⁴¹ The light microscopy findings of the glomeruli and the locations of immune complex deposition determine the pattern of ICGN.¹⁴² In general, the following classifications of ICGN are used in dogs: *membranous ICGN* – displays presence of subepithelial electron dense immune complex deposits without significant glomerular hypercellularity, and the deposits eventually lead to remodeling and thickening of the capillary walls; *membranoproliferative ICGN* – displays diffuse global capillary wall

thickening and mesangial expansion due to the presence of electron dense immune complex deposits in subendothelial and mesangial zones, increased production of mesangial matrix/mesangial hypercellularity, endocapillary hypercellularity (due to numerous circulating leukocytes and hypertrophied endothelium), and mesangial interpositioning into the capillary wall that eventually results in a glomerular basement membrane with double contours; *mesangioproliferative ICGN* – displays mesangial expansion/hypercellularity with electron dense immune complex deposits limited to the mesangial zones; and *mixed ICGN* – displays features that are a mixture of the ICGN forms described (e.g., large numbers of immune complexes in more than one location of the glomerulus).¹⁴³⁻¹⁴⁵ Although ICGN is a common cause of glomerular disease in dogs, other common causes of glomerular disease include amyloidosis, focal segmental glomerulosclerosis (FSGS) (e.g., intraglomerular hypertension), with structural abnormalities and maldevelopment less commonly observed.^{11,141}

Standard therapy for renal disease rarely completely resolves renal injury in cases of ICGN; therefore, treatment recommendations include attempting to identify and correct any underlying disease process that may be inciting the production of antigen-antibody complexes and suppressing the immune system to decrease immune complex formation.^{141,146} In human studies, immunosuppressive treatment appears effective in many cases of glomerulonephritis.¹⁴⁷⁻¹⁵² While there are no placebo controlled, biopsy-documented studies to support the use of immunosuppressives in dogs with ICGN, immunosuppressive therapy has anecdotally been beneficial in some cases of canine ICGN.^{146,153} A number of contraindications exist for the use of immunosuppressives in

some dogs. Ideally, evidence of an active immune-mediated mechanism promoting glomerular disease in the kidney would be obtained prior to starting treatment.^{146,153}

Currently there are no non-invasive biomarkers available for dogs that can diagnose the presence of ICGN. Therefore, a renal biopsy with light microscopy (LM), transmission electron microscopy (TEM), and immunofluorescence (IF) analysis is needed to establish the presence of immune complexes in the glomeruli and to guide therapy.¹⁴⁶ For many canine patients, a renal biopsy is not an option due to patient health, financial, or other constraints.¹⁵³ While use of minimally invasive biomarkers is not expected to replace all of the information gleaned from a renal biopsy, identification of biomarkers that suggest the presence of ICGN would be extremely helpful for guiding therapy when a biopsy cannot be performed.

A previous study found that several minimally invasive urinary biomarkers might be useful for identification of ICGN without the need for biopsy.¹² In particular, markedly increased urine immunoglobulin M (IgM), a high molecular weight (HMW) protein suggestive of glomerular damage, and urine N-acetyl- β -D-glucosaminidase (NAG), a lysosomal enzyme suggestive of tubular and potentially glomerular damage, were associated with ICGN.^{12,15,93} The goal of the current study was to evaluate these biomarkers in a larger cohort of dogs with corresponding renal biopsies in order to provide a more accurate assessment of the association of urinary IgM and NAG with the presence of ICGN in dogs. Additionally, we aimed to determine the ability of these biomarkers to predict prognosis in dogs with ICGN and determine whether they differed according to ICGN subtype.

Materials and Methods

Sample Collection and Processing of Samples

Dogs with Renal Disease

Urine supernatant, serum, and kidney tissues collected from dogs were submitted to the International Veterinary Renal Pathology Service (IVRPS) for diagnostic purposes between January 2008 and September 2014. All samples were collected and processed as previously described.¹² Cases were categorized as having an inactive urine sediment, hematuria (grossly or microscopically (> 5 red blood cells per $40\times$ field)), pyuria (> 5 white blood cells per $40\times$ field)/bacteriuria (based on culture or sediment findings of bacteria), or both hematuria and pyuria/bacteriuria, identified either on the submitted sample, if available, or within 4 weeks of the biopsy. Cases were excluded from analysis if an active urine sediment was identified. Renal biopsies were routinely processed for LM and TEM as previously described.¹¹ Renal disease was diagnosed based on presence of persistent proteinuria, azotemia, or both. Dogs were determined to have CKD (based on evidence of renal disease for ≥ 3 months or chronic changes on renal ultrasound or histology), acute kidney injury (AKI), AKI superimposed on CKD, or insufficient information to determine renal disease chronicity.

Sample Collection and Processing of Control Samples from Healthy Dogs

Urine and serum samples were collected from 9 client owned dogs and one unaffected (normal) dog from a colony with X-Linked Hereditary Nephropathy (XLHN)¹⁵⁴ that were healthy and had inactive urine sediments. Client consent was

obtained for the client-owned dogs. The dogs were determined to be healthy based on history, physical examination, complete blood count, serum chemistry, complete urinalysis, and urine protein:creatinine (UPC). Urine was collected as voided samples or by cystocentesis, and urinalysis was performed within 2 hours of collection. Urine supernatant and serum were stored at -80°C prior to analysis.

Histopathological Analysis and Scoring

Renal biopsies were assessed by one nephropathologist (REC), and biopsies were assigned an overall histologic diagnosis as previously described,¹² including: immune complex-mediated glomerulonephritis (ICGN), glomerulosclerosis, amyloidosis, other glomerulonephropathies, and tubular disease. “Other nephropathies” included those that affected the entire kidney, such as juvenile nephropathy (e.g., renal maldevelopment), as well as glomerulopathies, such as glomerulocystic atrophy, glomerular lipidosis, and glomerular basement membrane and podocyte abnormalities without immune complex deposition. For cases diagnosed as ICGN, subcategories were determined as described in the introduction, including membranous, membranoproliferative, mesangioproliferative, or mixed ICGN. Cases that were not diagnostic were excluded from further analysis.

Biomarkers

Commercial assay kits for IgM^l and NAG^m were used and have previously been validated in canine urine.^{12,37} Biomarkers were measured in duplicate. Each sample received ≤ 5 freeze-thaw cycles. Conventional biomarkers (serum creatinine (sCr),^{n,o} UPC,^{n,o,p} and urine specific gravity (USG)^q) and novel biomarkers (urine and serum IgM (uIgM and sIgM), and urine NAG (uNAG)) were measured in our laboratory. Urine biomarker concentrations were normalized to urine creatinine concentration (e.g., uIgM/c). Using the spot sample approach,¹²⁹ fractional excretion (FE) of IgM (IgM_FE) was calculated using the formula:

$$FE_{\text{analyte}} = (\text{Analyte}_{\text{urine}} / \text{Analyte}_{\text{serum}}) \times (\text{sCr} / \text{Creatinine}_{\text{urine}}) \times 100.$$

Survival Data

Owners and veterinarians for each dog were contacted between 2 months to 8 years post-renal biopsy. If the dog had died since biopsy, the amount of time to death post-biopsy, type of death (spontaneous versus humane euthanasia), and cause of death or decision to euthanize (e.g., renal disease progression) were recorded.

^l Dog IgM ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery, TX

^m N-acetyl- β -D-glucosaminidase (NAG) Assay, Diazyme Laboratories, Poway, CA

ⁿ Creatinine Liquicolor Test (Endpoint), Stanbio Laboratory, Boerne, TX

^o Siron Clinical Chemistry Analyzer, Stanbio Laboratory, Boerne, TX

^p Protein, Micro Liquicolor Test (CSF and Urine), Stanbio Laboratory, Boerne, TX

^q Rhino VET360 Veterinary Clinical Refractometer, Reichert Technologies, Depew, NY

Statistical Analysis

Biomarker values were not normally distributed; thus, non-parametric analyses were performed.

Comparison of Biomarkers Between Dogs with Inactive versus Active Urine Sediments

Although only cases with inactive urine sediments were used for most analyses, we were interested in determining if biomarker values differed significantly between dogs with inactive and active urine sediments. Using the 2-sample Wilcoxon rank sum (Mann-Whitney) test, urine protein biomarkers (UPC, uIgM, uIgM/c, IgM_FE, uNAG, and uNAG/c) were compared between dogs with inactive urine sediments and dogs with hematuria, pyuria/bacteriuria, or both. Further analyses excluded cases with active urine sediments.

Comparison of Biomarkers Between Healthy Dogs and Dogs with Renal Disease

Using the 2-sample Wilcoxon rank sum test, conventional biomarkers (sCr, USG, and UPC) and novel urine protein biomarkers (uIgM, uIgM/c, IgM_FE, uNAG, and uNAG/c) were compared between healthy dogs and dogs with ICGN and non-ICGN renal disease to determine biomarker differences between healthy and ill dogs. These same comparisons were also performed to determine differences between dogs with ICGN and non-ICGN and among the 4 different categories of ICGN.

Prediction of ICGN

Receiver operator characteristic (ROC) analysis was performed to determine the area under the ROC curve (AUC), and sensitivities and specificities were calculated for each urine protein biomarker for the ability to detect renal disease due to ICGN. These same determinations were made based on grouping of the data according to presence or absence of azotemia and for dogs biopsied for suspicion of glomerular disease (i.e., if the clinical history mentioned suspicion of nephritic syndrome, nephrotic syndrome, or glomerulonephritis, UPC was > 2 , or persistent renal proteinuria was identified in cases without obvious non-glomerular diseases). The presence of ICGN on histology was set as the gold standard (1 was positive presence of disease, 0 was otherwise). Cutoff values for conventional and novel biomarkers were determined based on that which maximized the sum of the sensitivity and specificity.

Survival Analysis

For each urine protein biomarker, a survival model was fit using a Cox semi-parametric model (accounting for the biomarker and age as covariates) to estimate median time to death due to renal disease post-biopsy. Hazard ratios (HR) were used to describe the association of the biomarker and age with risk of death due to renal disease.

All statistical analyses were carried out using Stata version 11, setting $P < 0.05$.[†]

[†] Stata Corp. LP, College Station, TX

Results

Dogs and Samples

Samples were initially analyzed from 267 dogs with renal disease, most of which were proteinuric. Urinalysis results were available on the submitted urine samples or on a sample within 4 weeks of biopsy collection in 71% of cases. Of these, 32 had hematuria, 4 had pyuria/bacteriuria, and 10 had both hematuria and pyuria/bacteriuria. These were excluded from all analyses except the comparison of active vs inactive sediments. An additional 8 cases were non-diagnostic on histologic analysis of the submitted renal biopsy and were also excluded. The remaining 213 cases represented a wide variety of breeds. Retriever breeds (Labrador retrievers, Golden retrievers, and mixes) were the most commonly represented with 37 dogs (17.4%), followed by Yorkshire terriers and Yorkshire terrier mixes with 12 dogs (5.6%). Ninety spayed females (42.3%), 73 castrated males (34.3%), 31 intact males (14.6%), and 19 intact females (8.9%) were included in the study. Nine dogs (4.3%) were < 1 year of age; 60 dogs (28.4%) were ≥ 1 to ≤ 5 years; 117 dogs (55.5%) were ≥ 5 to ≤ 10 years; and 25 dogs (11.9%) were ≥ 10 years.

Presence of kidney damage was diagnosed by clinicians based on persistent proteinuria in 105 (49.3%) dogs, azotemia in 22 (10.3%) dogs, and both proteinuria and azotemia in 86 (40.4%) dogs. Based on known timeframe of disease symptoms and/or presence of chronicity seen on ultrasound and renal histology, CKD was confirmed for 225 (87.6%) dogs. Seven dogs (2.7%) had AKI superimposed on CKD, 5 dogs (2%) had

AKI, and in 20 dogs (7.8%) there was not enough information available to determine the chronicity of the renal disease.

Outcome/prognostic information was available for 120 (56.3%) of the dogs with renal disease included in the analysis. Cause of death following biopsy was ascertained for 79 dogs of which 58 died or were euthanized due to progression of renal disease. For these 58 dogs, median time to death due to renal disease following biopsy (not including samples obtained at necropsy (6 samples)) was 183 days (range: 2 – 1,460 days).

Histopathological Analysis

Figure 9A illustrates the percentages of different histologic diagnoses (ICGN, glomerulosclerosis, amyloidosis, other glomerulonephropathies, and primary tubular disease) in the 213 dogs included in the analysis. Figure 9B illustrates the percentages of the different categories of ICGN (membranous (MGN), membranoproliferative (MPGN), mesangioproliferative, and mixed) in the 85 dogs that were diagnosed with ICGN.

Comparison of Biomarkers Among Dogs

Healthy Dogs versus Dogs with Renal Disease

All urine protein biomarkers were significantly higher in dogs with ICGN compared to healthy dogs, while all except for uNAG were greater in dogs with non-ICGN renal disease compared to healthy dogs (Table 5). sCr was significantly lower and USG was significantly higher in healthy dogs compared to dogs with non-ICGN renal

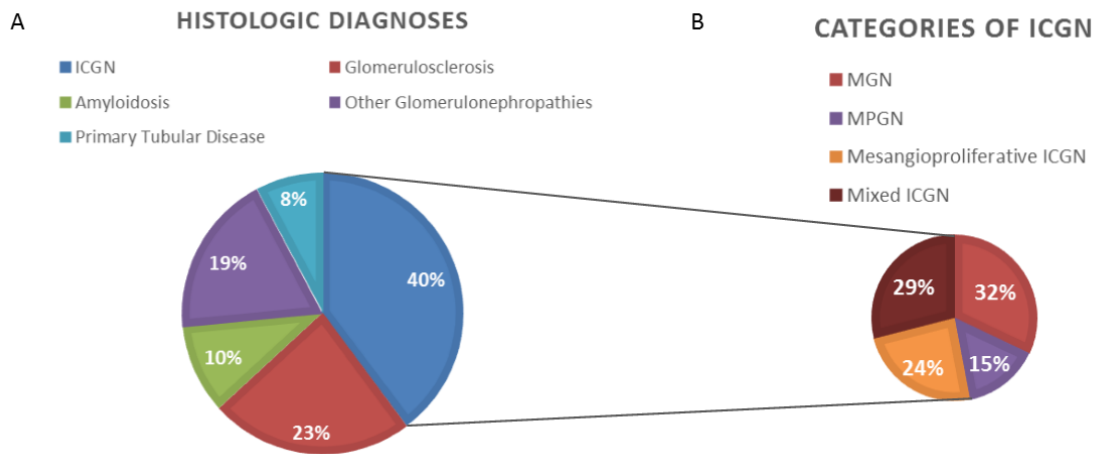


Figure 9. A) Proportion of 213 dogs included for histologic analysis of renal biopsies that were diagnosed with immune complex-mediated glomerulonephritis (ICGN), glomerulosclerosis, amyloidosis, other glomerulonephropathies, or primary tubular disease; B) proportion of the 85 ICGN dogs that had membranous glomerulonephritis (MGN), membranoproliferative glomerulonephritis (MPGN), mesangioproliferative ICGN, or mixed ICGN.

disease. Neither sCr nor USG was significantly different between healthy dogs and dogs with ICGN. For most biomarkers, there was some overlap in values for dogs without evidence of renal disease and those with renal disease due to ICGN or non-ICGN. However, for UPC and uIgM/c, there was no overlap in values between healthy dogs and those with ICGN.

Although cases with active urine sediments were excluded from most analyses, we were interested in determining if there was an evident difference in biomarker values in dogs with renal disease with and without active urine sediments. Table 6 displays the median and range of each urinary biomarker categorized by whether the case had an inactive urine sediment, hematuria, pyuria/bacteriuria, or both hematuria and

pyuria/bacteriuria. All 3 of the IgM biomarkers (uIgM, uIgM/c, and IgM_FE) were significantly increased in cases with hematuria and with both hematuria and pyuria/bacteriuria. NAG and UPC were not significantly different among groups.

Non-ICGN versus ICGN Dogs

All urine protein biomarkers were significantly higher in dogs with renal disease due to ICGN versus non-ICGN, although substantial overlap was present for all biomarkers between these categories (Table 5 and Figures 10 - 15). Additionally, USG was significantly higher in ICGN versus non-ICGN dogs, while there was no significant difference in sCr between the categories.

Comparison Between ICGN Classifications

Several biomarkers displayed significant differences between categories of ICGN (Table 7). Of particular interest, uIgM, uIgM/c, and IgM_FE were significantly greater in MPGN than the majority of other ICGN categories. UPC, uNAG, and uNAG/c were significantly greater in both MGN and mixed ICGN compared with mesangioproliferative ICGN.

Table 5. Comparison of biomarkers in healthy dogs versus dogs with ICGN and non-ICGN renal disease based on analysis using the 2-sample Mann-Whitney test.

	Healthy		Non-ICGN		ICGN	
	N	Median (Range)	N	Median (Range)	N	Median (Range)
sCr (mg/dl)	9	1.0 (0.7 - 1.3)	128	1.5 (0.3 - 21.8) ^a	81	1.5 (0.3 - 6.7)
USG	9	1.022 (1.012 - 1.054)	128	1.015 (1.003 - 1.048) ^a	85	1.020 (1.005 - 1.046) ^c
UPC	9	0.0 (0.0 - 0.2)	128	3.7 (0.0 - 31.6) ^a	85	8.0 (0.4 - 36.8) ^{b,c}
uIgM (ng/ml)	9	0.7 (0.5 - 1.2)	128	3.2 (0.1 - 44.5) ^a	85	13.6 (1.2 - 403.8) ^{b,c}
uIgM/c (µg/mg)	9	0.3 (0.1 - 0.8)	128	4.1 (0.3 - 28.3) ^a	85	14.4 (2.2 - 557.0) ^{b,c}
IgM_FE (%)	9	0.000 (0.000 - 0.000)	100	0.002 (0.000 - 0.172) ^a	64	0.006 (0.000 - 0.089) ^{b,c}
uNAG (U/L)	8	5.8 (2.0 - 21.6)	128	7.6 (0.1 - 59.9)	85	24.4 (1.6 - 159.5) ^{b,c}
uNAG/c (U/g)	8	1.5 (1.0 - 5.9)	128	9.9 (0.4 - 80.4) ^a	85	24.1 (3.0 - 427.7) ^{b,c}

P < 0.05 significantly different between ^ahealthy and non-ICGN dogs; ^bhealthy and ICGN dogs; ^cnon-ICGN and ICGN dogs; sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine; uIgM, urine immunoglobulin M; uIgM/c, urine immunoglobulin M/creatinine; IgM_FE, fractional excretion of immunoglobulin M; uNAG, urine N-acetyl-β-D-glucosaminidase; uNAG/c, urine N-acetyl-β-D-glucosaminidase/creatinine; ICGN, immune complex-mediated glomerulonephritis.

Table 6. Comparison of urinary biomarkers in dogs with inactive versus active urine sediments based on analysis using the 2-sample Mann-Whitney test.

	Inactive Sediment		Hematuria		Pyuria/Bacteriuria		Hematuria and Pyuria/Bacteriuria	
	N	Median (Range)	N	Median (Range)	N	Median (Range)	N	Median (Range)
UPC	213	5.1 (0.0 - 36.8)	30	7.0 (0.7 - 26.7)	4	7.0 (0.5 - 8.9)	10	6.0 (1.3 - 23.6)
uIgM (ng/ml)	213	5.1 (0.1 - 403.8)	30	8.9 (0.4 - 335.2) ^a	4	2.5 (1.1 - 3.4)	10	12.0 (2.7 - 163.8) ^b
uIgM/c (µg/mg)	213	6.5 (0.3 - 557.0)	30	10.4 (0.8 - 546.1) ^a	4	4.3 (2.4 - 6.3)	10	31.2 (5.3 - 240.8) ^b
IgM_FE (%)	164	0.003 (0.000 - 0.172)	17	0.010 (0.002 - 0.163) ^a	2	0.005 (0.003 - 0.007)	8	0.045 (0.006 - 0.122) ^b
uNAG (U/L)	213	12.6 (0.1 - 159.5)	30	21.0 (1.3 - 122.0)	4	11.2 (3.3 - 27.1)	10	17.3 (2.5 - 48.1)
uNAG/c (U/g)	213	13.9 (0.4 - 427.7)	30	16.0 (2.6 - 110.3)	4	19.6 (10.3 - 39.2)	10	34.1 (6.4 - 92.5)

P < 0.05 significantly different between ^ainactive sediment and hematuria; ^binactive sediment and hematuria/pyuria/bacteriuria; UPC, urine protein:creatinine; uIgM, urine immunoglobulin M; uIgM/c, urine immunoglobulin M/creatinine; IgM_FE, fractional excretion of immunoglobulin M; uNAG, urine N-acetyl-β-D-glucosaminidase; uNAG/c, urine N-acetyl-β-D-glucosaminidase/creatinine.

Table 7. Comparison of biomarkers in different ICGN classifications based on analysis using the 2-sample Mann-Whitney test.

	Membranous		Membranoproliferative		Mesangioproliferative		Mixed	
	N	Median (Range)	N	Median (Range)	N	Median (Range)	N	Median (Range)
sCr (mg/dl)	25	1.4 (0.3 - 6.7)	13	2.0 (0.7 - 3.3) ^d	20	1.1 (0.6 - 3.2) ^d	23	1.7 (0.4 - 4.5)
USG	27	1.021 (1.005 - 1.041)	13	1.018 (1.010 - 1.045)	20	1.018 (1.005 - 1.044) ^f	25	1.028 (1.015 - 1.046) ^f
UPC	27	9.8 (0.5 - 20.9) ^b	13	7.4 (2.5 - 25.2)	20	5.0 (0.4 - 15.3) ^{b,f}	25	9.1 (2.8 - 36.8) ^f
uIgM (ng/ml)	27	13.1 (1.2 - 97.1) ^a	13	42.3 (5.1 - 403.8) ^{a,d}	20	7.0 (1.8 - 33.5) ^{d,f}	25	19.9 (2.5 - 226.6) ^f
uIgM/c (μg/mg)	27	9.3 (2.4 - 48.3) ^{a,c}	13	52.3 (10.8 - 557.0) ^{a,d,e}	20	9.5 (2.4 - 61.3) ^{d,f}	25	18.3 (2.2 - 129.5) ^{e,c,f}
IgM_FE (%)	16	0.004 (0.000 - 0.025) ^{a,c}	13	0.018 (0.002 - 0.089) ^{a,d}	17	0.003 (0.001 - 0.027) ^{d,f}	18	0.017 (0.001 - 0.064) ^{c,f}
uNAG (U/L)	27	44.3 (4.4 - 130.1) ^b	13	18.4 (4.1 - 159.5)	20	12.3 (1.6 - 65.5) ^{b,f}	25	35.7 (7.0 - 139.4) ^f
uNAG/c (U/g)	27	30.3 (7.1 - 131.3) ^b	13	17.4 (7.0 - 124.7)	20	15.2 (3.0 - 55.3) ^{b,f}	25	30.2 (4.2 - 427.7) ^f

P < 0.05 significantly different between ^amembranous and membranoproliferative ICGN; ^bmembranous and mesangioproliferative ICGN; ^cmembranous and mixed ICGN; ^dmembranoproliferative and mesangioproliferative ICGN; ^emembranoproliferative and mixed ICGN; ^fmesangioproliferative and mixed ICGN; sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine; uIgM, urine immunoglobulin M; uIgM/c, urine immunoglobulin M/creatinine; IgM_FE, fractional excretion of immunoglobulin M; uNAG, urine N-acetyl-β-D-glucosaminidase; uNAG/c, urine N-acetyl-β-D-glucosaminidase/creatinine; ICGN, immune complex-mediated glomerulonephritis.

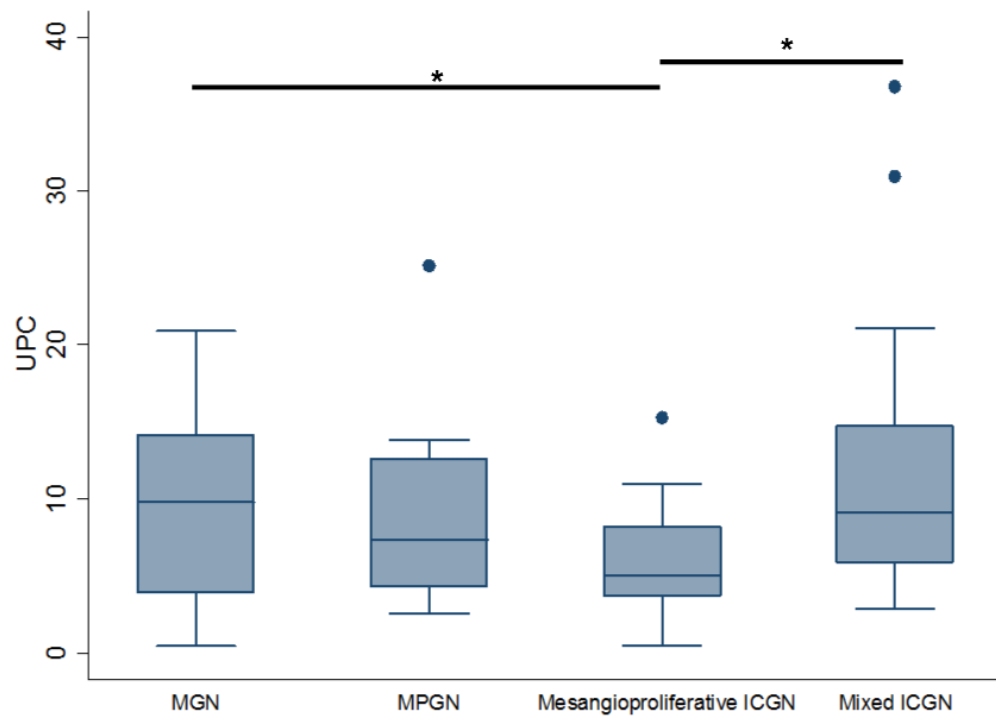


Figure 10. Comparison of urine protein:creatinine (UPC) between different categories of immune complex-mediated glomerulonephritis (ICGN). * $P < 0.05$ between categories of ICGN). MGN, membranous glomerulonephritis; MPGN, membranoproliferative glomerulonephritis.

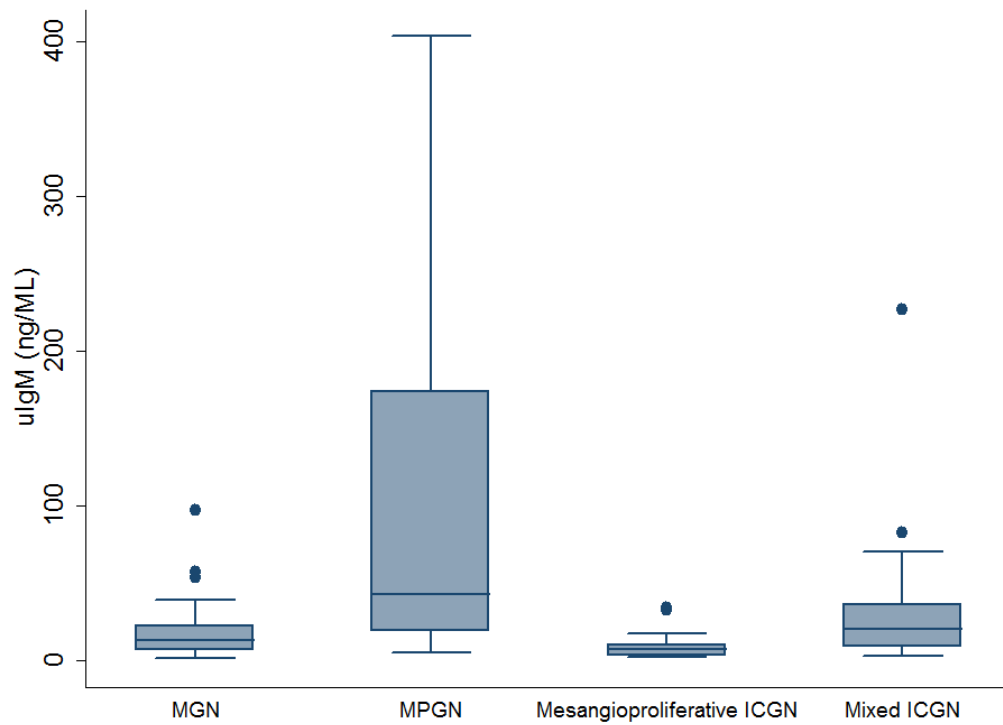


Figure 11. Comparison of non-normalized urine immunoglobulin M (uIgM) between different categories of immune complex-mediated glomerulonephritis (ICGN). * $P < 0.05$ between categories of ICGN). MGN, membranous glomerulonephritis; MPGN, membranoproliferative glomerulonephritis; GN, glomerulonephritis.

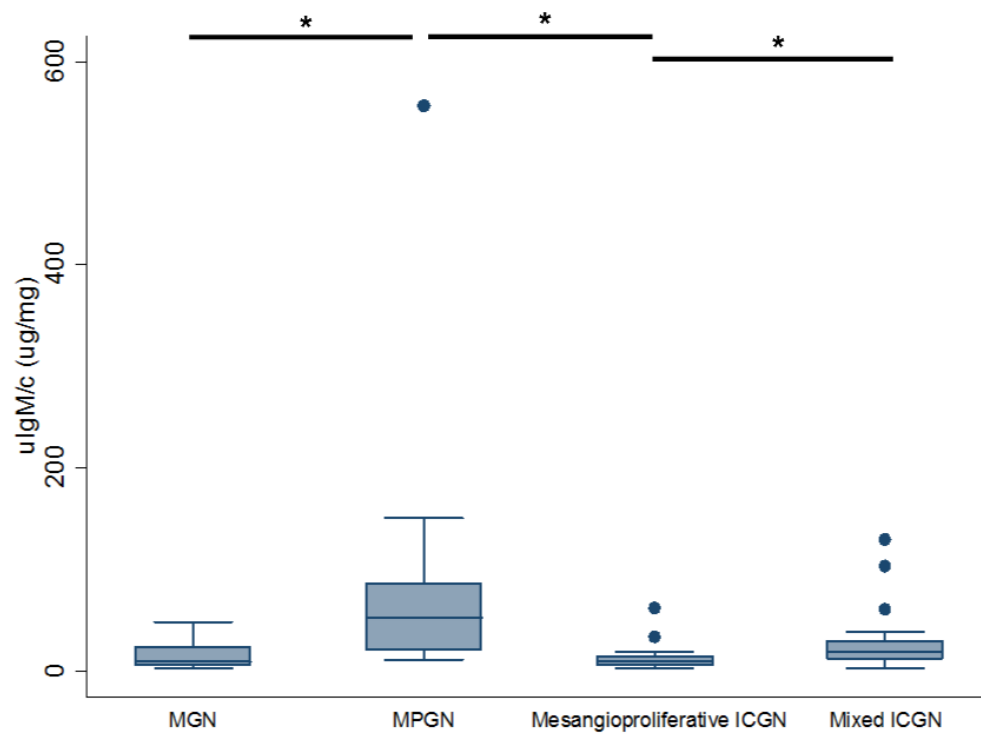


Figure 12. Comparison of urine immunoglobulin M/creatinine (uIgM/c) between different categories of immune complex-mediated glomerulonephritis (ICGN). * $P < 0.05$ between categories of ICGN). MGN, membranous glomerulonephritis; MPGN, membranoproliferative glomerulonephritis.

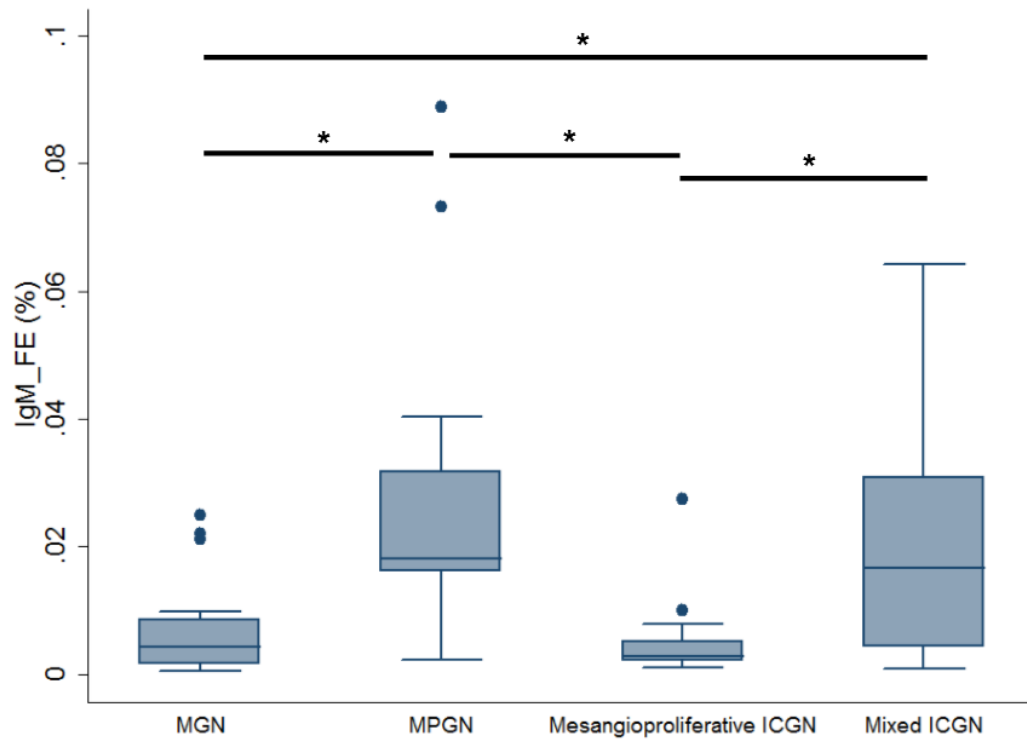


Figure 13. Comparison of the fractional excretion of immunoglobulin M (IgM_FFE) between different categories of immune complex-mediated glomerulonephritis (ICGN). *P < 0.05 between categories of ICGN). MGN, membranous glomerulonephritis; MPGN, membranoproliferative glomerulonephritis; GN, glomerulonephritis.

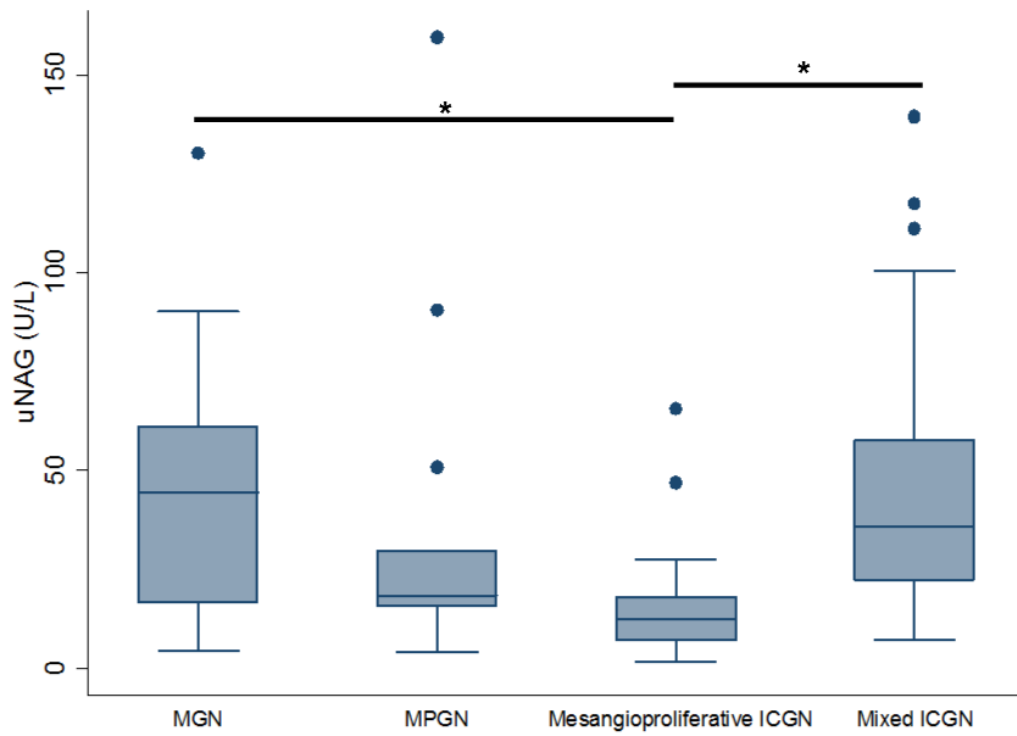


Figure 14. Comparison of the non-normalized urine N-acetyl- β -D-glucosaminidase (uNAG) between different categories of immune complex-mediated glomerulonephritis (ICGN). *P < 0.05 between categories of ICGN). MGN, membranous glomerulonephritis; MPGN, membranoproliferative glomerulonephritis; GN, glomerulonephritis.

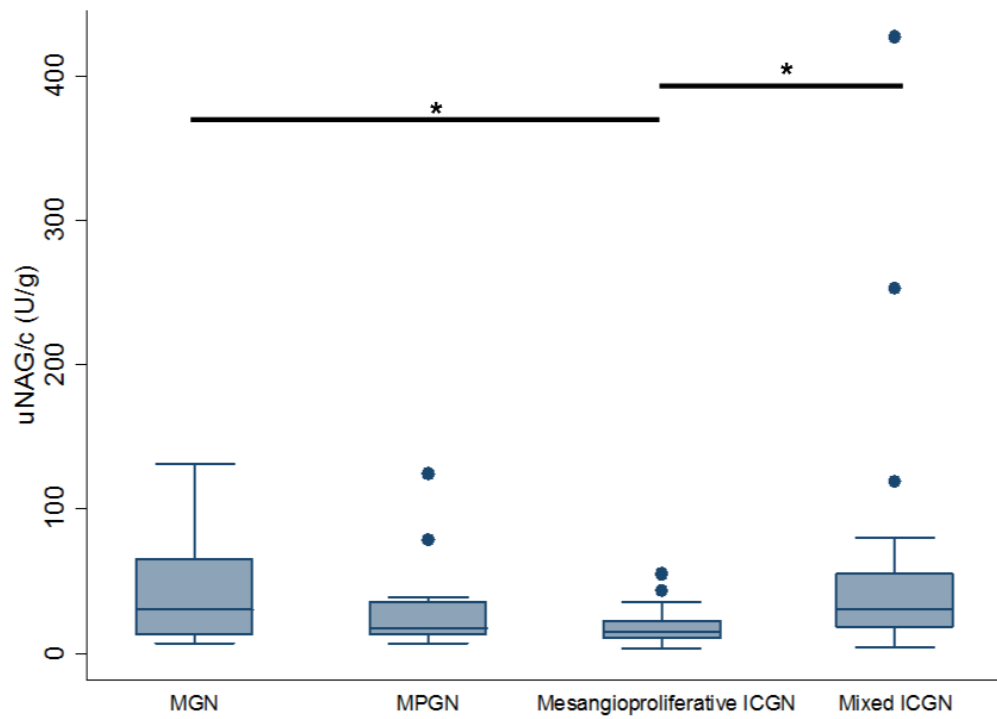


Figure 15. Comparison of the urine N-acetyl- β -D-glucosaminidase/creatinine (uNAG/c) between different categories of immune complex-mediated glomerulonephritis (ICGN). *P < 0.05 between categories of ICGN). MGN, membranous glomerulonephritis; MPGN, membranoproliferative glomerulonephritis; GN, glomerulonephritis.

Prediction of ICGN

To determine which urinary protein biomarkers might be useful indicators of ICGN in dogs with proteinuric renal disease, ROC analysis and sensitivity and specificity calculations were performed for each biomarker, with diagnosis of ICGN based on kidney biopsy set as the gold standard. Table 8 describes the ROC AUC for each biomarker's ability to diagnose the presence of ICGN. When all cases were included in the analysis regardless of stage of disease progression, uIgM demonstrated the highest AUC (0.8433), with a sensitivity of 83.5% and a specificity of 75.0% based on a cutoff of > 5.3 ng/mL. Cases were also grouped by progression of disease as determined by whether the dog was non-azotemic (International Renal Interest Society (IRIS) stage 1) or azotemic (IRIS stages 2, 3, or 4) at the time of sample submission. AUCs demonstrated a mild decrease for all urine protein biomarkers in non-azotemic dogs but a mild increase in azotemic dogs compared to the entire cohort of cases. In particular, specificity of uIgM/c increased to 89% in the azotemic cohort. Given the relatively high AUCs of uIgM and uIgM/c for detection of ICGN, we were interested in determining changes in specificity at cutoff values higher than those which maximized the sum of sensitivity and specificity, since a specific non-invasive diagnosis of ICGN would be most helpful for clinicians to confidently treat with immunosuppressive drugs. When the median values of uIgM and uIgM/c in ICGN dogs (13.6 ng/ml and 14.4 μ g/mg, respectively (Table 5)) were used as cutoff values in ROC analysis, specificities of uIgM and uIgM/c for detection of ICGN in all dogs increased to 93.0% and 90.6%, respectively. However, sensitivity decreased to 50.6% for both biomarkers. When these

same cutoff values for uIgM and uIgM/c were used in the azotemic group, specificities of uIgM and uIgM/c for detection of ICGN were 94.5% and 90.4% respectively, while sensitivities were 62.8% and 64.7%, respectively. In the non-azotemic group, specificities of uIgM and uIgM/c for detection of ICGN were both 90.9%, and sensitivities were 32.4% and 29.4%, respectively.

Since clinicians will only be suspecting ICGN when they have a high clinical suspicion for glomerular disease, we also performed the same calculations to determine the ability of biomarkers to detect ICGN only in those dogs biopsied for suspicion of glomerular disease (n = 178 dogs). AUCs for all urine protein biomarkers slightly decreased when this truncated population was used (Table A-11); however, when the median values of uIgM and uIgM/c in ICGN dogs (13.6 ng/ml and 14.4 µg/mg, respectively) were used as cutoff values in ROC analysis, specificities of uIgM and uIgM/c for detection of ICGN in dogs with suspicion for glomerular disease were between 89 – 93% depending on presence of azotemia.

Table 8. Ability of urinary protein biomarkers to predict ICGN in all dogs and based on presence of azotemia. Areas under the curve, optimal cutoff values, and corresponding sensitivities and specificities, as determined by receiver operator characteristic analysis, are displayed.

	All dogs (N=213)		Non-Azotemic Dogs (N= 89)		Azotemic Dogs (N= 124)	
	AUC	Cutoff (Sens %, Spec %)	AUC	Cutoff (Sens %, Spec %)	AUC	Cutoff (Sens %, Spec %)
UPC	0.7199	>2.1 (96.5, 38.3)	0.6193	>2.1 (94.1, 27.3)	0.7826	>5.3 (74.5, 74.0)
uIgM (ng/ml)	0.8433	>5.3 (83.5, 75.0)	0.7422	>5.3 (79.4, 65.5)	0.9065	>4.5 (92.2, 76.7)
uIgM/c (µg/mg)	0.8199	>7.3 (76.5, 71.9)	0.7604	>6.4 (79.4, 70.9)	0.8590	>13.4 (68.6, 89.0)
IgM_FE (%)	0.7245	>0.007 (48.4, 86.0)	0.6855	>0.001 (80.8, 51.1)	0.7799	>0.007 (73.7, 74.5)
uNAG (U/L)	0.7989	>11.2 (80.0, 65.6)	0.6618	>27.6 (44.1, 89.1)	0.8927	>11.1 (88.2, 78.1)
uNAG/c (U/g)	0.7691	>14.9 (72.9, 69.5)	0.6957	>23.5 (52.9, 81.8)	0.8171	>14.9 (78.4, 75.3)

AUC, area under the curve; ROC, receiver operator characteristic, ICGN, Immune Complex-Mediated Glomerulonephritis; IRIS, International Renal Interest Society; Sens, sensitivity; Spec, specificity; UPC, urine protein:creatinine; uIgM, urine immunoglobulin M; uIgM/c, urine immunoglobulin M/creatinine; IgM_FE, fractional excretion of immunoglobulin M; uNAG, urine N-acetyl-β-D-glucosaminidase; uNAG/c, urine N-acetyl-β-D-glucosaminidase/creatinine.

Survival Analysis

Increases in UPC and IgM_FE were significantly associated with increased risk of death due to renal disease according to Cox survival models including age as a covariate. UPC had an HR of 1.06 (95% confidence interval (CI) = 1.02 – 1.10; P = 0.004) and IgM_FE had an HR of 1.34 (95% CI = 1.12 – 1.60; P = 0.002). An increase in UPC of 1 unit increased the risk of death by 6%, and an increase in IgM_FE of 0.01% increased the risk of death by 34%. Age also had a significant association with the risk of death due to renal disease when combined in survival models as a covariate for UPC and IgM_FE (a 1 year increase in age increased the risk of death associated with these biomarkers). Figures 16 and 17 illustrate the probability of survival modeled in a dog of median age at different starting values of UPC and IgM_FE.

Presence of ICGN was not associated with an increased risk of death due to renal disease (HR of 0.958 (95% CI = 0.551 – 1.67; P = 0.880)). Dogs with ICGN for which survival data was available had a median time to death due to renal disease of 192 days (range: 3 – 1,190 days) and dogs with non-ICGN renal disease had a median time to death of 179 days (range: 2 – 1,460 days). The median and range for time to death due to renal disease for dogs in each non-ICGN subtype are as follows: amyloidosis: 183 (2 – 305) days; glomerulosclerosis: 194 (2 – 1,460) days; other glomerulonephropathies: 66 (3 – 1,349) days; primary tubular disease: 328 (22 – 634) days. Median times to death due to renal disease for dogs in each ICGN group were as follows: MGN: 19 days (range: 3-122 days); MPGN: 244 days (range: 7-1,098 days); mesangioproliferative ICGN: 758 days (range: 201-1,190 days); and mixed ICGN: 183 days (range: 8-1,098

days). The low number of cases in each group (5 to 7 dogs per ICGN group) precluded the ability to perform an accurate analysis of significant differences between the median survival times in different ICGN groups.

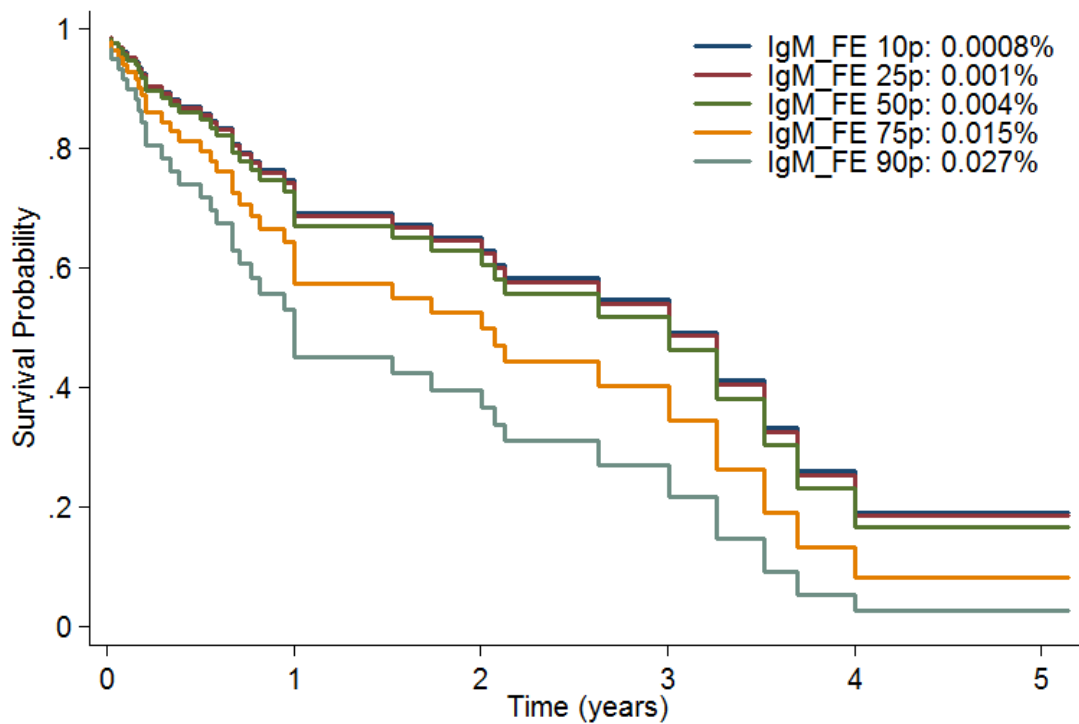


Figure 16. Probability of survival for a dog at median age (7 years) at different starting values of fractional excretion of immunoglobulin M (IgM_FE). 10p: 10th percentile; 25p: 25th percentile; 50p: 50th percentile; 75p: 75th percentile; 90p: 90th percentile.

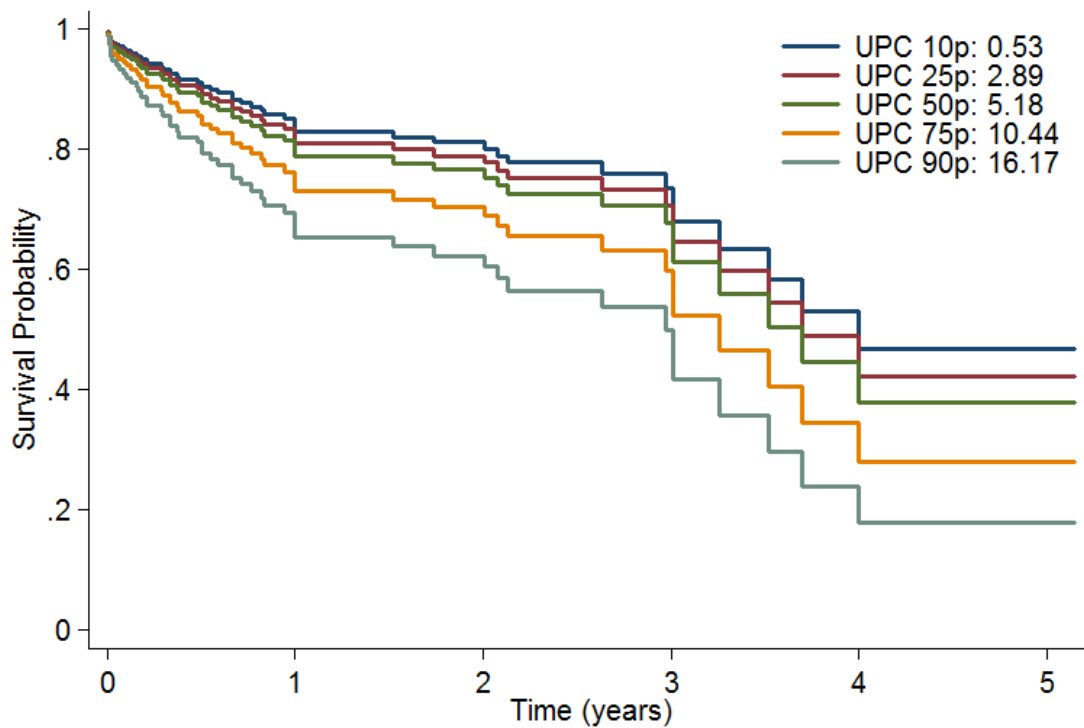


Figure 17. Probability of survival for a dog at median age (7 years) at different starting values of urine protein:creatinine (UPC). 10p: 10th percentile; 25p: 25th percentile; 50p: 50th percentile; 75p: 75th percentile; 90p: 90th percentile.

Discussion

This retrospective study expands upon previous work exploring the correlation of several urinary biomarkers with the degree of glomerular and TI damage on renal histology in dogs with naturally occurring proteinuric CKD and the ability of these biomarkers to detect specific types of renal diseases.¹² Previously, urinary IgM and NAG were associated with the presence of ICGN and had moderately high sensitivities and specificities for ICGN detection. The current study was designed to further explore the ability of these 2 urinary protein biomarkers as indicators of ICGN in a larger cohort of dogs with renal disease (n = 213 vs 180) with stricter criteria for exclusion. This study confirmed that most of the novel and conventional biomarkers measured were significantly greater in dogs with renal disease due to ICGN or non-ICGN causes than in healthy dogs, and UPC and urinary IgM and NAG were all significantly higher in dogs with ICGN compared to dogs with non-ICGN causes. Of the biomarkers evaluated, uIgM demonstrated the greatest ability to detect ICGN, particularly in azotemic dogs. IgM_FE and UPC were also significantly associated with reduced survival. Our findings support the supposition that urinary biomarkers might be useful to distinguish dogs with proteinuric renal disease induced by ICGN.

In the healthy dogs, measured values of uNAG/c were within the ranges previously reported for healthy dogs;^{101,103} however, normal values for urinary IgM in dogs (or any domestic species) have not been previously reported, and thus this study serves as the first report to confirm the low concentration of IgM in the urine of healthy dogs. To our knowledge, this current study and our previous study are the only ones

exploring the use of urinary IgM as a biomarker in dogs with CKD;¹² however, there is precedence for its use in human medicine where increased urinary IgM excretion was associated with increased risk of renal failure and death in people with diabetic glomerulonephropathy.^{130,131}

It is interesting that neither sCr nor USG were significantly different between healthy dogs and dogs with ICGN despite finding significant differences between healthy and non-ICGN dogs. This finding emphasizes the lack of sensitivity of these two conventional renal biomarkers for detection of kidney disease and the importance of performing a full urinalysis (including detection of proteinuria) with UPC determination if indicated rather than relying on elevated sCr or decreased USG to detect development of significant renal disease in dogs. Indeed, based on observations from the IVRPS, MGN in dogs often only presents with proteinuria, and thus this subtype of ICGN would likely have unremarkable sCr and USG upon presentation.

Comparison of biomarkers between samples with inactive and active urinary sediments demonstrated that urinary IgM was significantly higher in dogs with concurrent renal disease and hematuria or both hematuria and pyuria/bacteriuria as compared with dogs having inactive urine sediments. Cases with only pyuria/bacteriuria did not demonstrate significantly higher urinary IgM or other biomarkers compared with cases with inactive urine sediments. Thus, it is likely that hematuria contributes to the elevation in urinary IgM rather than pyuria/bacteriuria. However, because the dogs in this study also had renal disease, it is difficult to make more specific comments regarding elevated urinary biomarkers due to conditions that cause active urine

sediments. In general, urinary tract infections/inflammation or hematuria could compound the evaluation of urinary biomarkers, and currently, it is not recommended to perform urinary biomarker analysis if an active urine sediment is present.

A major goal of this study was to determine the ability of selected urinary biomarkers to distinguish between dogs with proteinuric renal disease due to ICGN versus non-ICGN causes. The novel biomarkers studied (IgM and NAG) and UPC were significantly greater in dogs with ICGN versus non-ICGN. Mechanistic studies have not yet been undertaken to understand possible causes for the differences in concentrations between the disease types. It is possible that in many dogs with ICGN, immune complexes generate greater damage to the glomerular filtration barrier and more significant loss of permselectivity compared to non-ICGN causes of proteinuric renal disease. Another possibility is that there might also be sloughing of cells or immune complex components containing IgM into the urine, which would contribute to the increased amount of urinary IgM. This latter theory would also explain the difference in sensitivity/specificity of uIgM and uIgM/c versus IgM_{FE}, which takes into account the serum IgM concentration.

Although, urinary IgM and NAG were significantly greater in dogs with ICGN, the ability to detect ICGN based on sensitivity and specificity of the tests was not perfect, particularly in non-azotemic dogs. Their performance decreased further when only dogs biopsied for suspicion of glomerular disease were considered in the analysis. However, this is likely because the non-ICGN CKD group included maldevelopment and primary tubular disease cases where these HMW proteins were expected to be low

in the urine. Sensitivities and specificities were improved in azotemic dogs, and thus, the utility of urinary IgM and NAG as early markers of ICGN in dogs might be more limited prior to onset of azotemia. However, at higher cutoff values for uIgM and uIgM/c, specificity of ICGN increased to $\geq 90\%$ in both non-azotemic and azotemic groups. Although sensitivities were poor at higher cutoff values, specificity is more important than sensitivity when considering treatment that could have detrimental effects. Based on our findings, dogs with markedly elevated uIgM or uIgM/c might be considered reasonable candidates for the cautious use of immunosuppressives without biopsy confirmation of disease. Furthermore, if multiple biomarkers are significantly elevated, this might provide greater evidence for the presence of ICGN, as a panel of biomarkers will likely be needed for best detection and distinction of the disease.

The AUCs for the ROC curve for uIgM and uIgM/c for detection of ICGN were greater than that for UPC, demonstrating the ability of novel biomarkers to provide predictive information beyond what is currently available with conventional tests. There is a significant drive for identifying non-invasive biomarkers in all species that can identify the presence of a particular glomerular disease without the need for biopsy for a number of reasons.¹⁵⁵⁻¹⁵⁸ A non-invasive biomarker might avoid the need for biopsy in an unstable patient, provide prognostic information that is beyond what is currently available, and help with tracking disease progression and response to therapy.¹⁵⁷ Of particular interest would be establishing a biomarker that would provide information on whether dogs with ICGN will benefit from immunosuppressive therapy. This will be an additional undertaking that will require significant exploration.

In addition to the biomarker differences observed between ICGN and non-ICGN groups, differences were also observed among the classifications of ICGN. Specifically, uIgM, uIgM/c, and IgM_FE were all significantly greater in dogs with MPGN versus MGN or mesangioproliferative ICGN. The severity of glomerular damage tended to be higher with MPGN vs. other forms, which could explain this finding. Because there is some disagreement on the ideal way to normalize urinary biomarkers, particularly in cases with acute damage, we evaluated the biomarkers both with and without normalization to urine creatinine concentration. In doing so, we found that the diagnostic performance for uIgM was greater than that for uIgM/c. The significance of this finding is unknown, but it supports that biomarkers should be analyzed both with and without normalization.

Our findings for the survival analysis in this study are somewhat consistent with our previous study in that IgM_FE is still significantly associated with an increased risk of death due to renal disease.¹² Additionally, we found that UPC was significantly associated with an increased risk of death due to renal disease in the current study, although the hazard ratio was minimally elevated. In our previous study, the association of UPC with risk of death approached statistical significance but the HR was very close to 1.0. For the current study, we employed slightly stricter exclusion guidelines for hematuria and pyuria/bacteriuria and included more animals with follow-up information, all of which are possible reasons for slightly different results. Our current findings are in line with a previous report that UPC is associated with survival time in dogs with CKD, although the increased risk is much less than what was reported in that study.¹²⁶ A

significant difference was not noted in the risk of death due to renal disease between dogs with ICGN or non-ICGN causes of CKD. Treatment protocols between dogs were not considered when outcome data was analyzed due to lack of consistent follow-up regarding protocols; however, certainly variable treatments and reduction of proteinuria in dogs could affect survival times. Therefore, future studies should consider categorizing outcome data by different treatment protocols. Although there were not enough cases to consider significant differences between risk of death and survival times dependent on ICGN groups, it is interesting that dogs in different ICGN groups had highly variable survival times, with median survival times for MGN and mesangioproliferative ICGN at opposite ends of the range (19 versus 758 days, respectively). A larger cohort of dogs with ICGN that includes outcome data is needed to determine if these survival time differences are consistent and significant prior to using this information for clinical purposes.

Because most of the dogs in this study had proteinuric CKD there was a bias for glomerular disease. However, our interest was in exploring biomarkers in dogs for more sensitive and specific detection of ICGN compared to other glomerular diseases. Thus, the cohort of dogs used would likely be representative of those being evaluated for ICGN. Additionally, a limitation of this study is that although most urine samples had a corresponding urinalysis (71%), many samples did not. Most samples were collected by internists at referral veterinary centers that ideally minimized the likelihood of sediment abnormalities. We were also very strict in our criteria of exclusion of cases with evidence of hematuria and/or pyuria/bacteriuria; however, it is possible that cases with

minimal hematuria or pyuria/bacteriuria were unknowingly included. Finally, for purposes of survival analysis, the inclusion of dogs that were euthanized is difficult to avoid in veterinary medicine and might skew survival times; however, it reflects the situation seen in the clinical setting.

In conclusion, urinary IgM and NAG have at least a moderate predictive ability for detecting ICGN in dogs with proteinuric CKD, particularly once azotemia has developed, and IgM_FE is a predictor of increased risk of death due to renal disease. While renal biopsy is certainly still advocated, these findings lend evidence to the hypothesis that urinary biomarkers could be used to support a diagnosis of ICGN so that patients might be more confidently started on a carefully monitored trial with immunosuppressive drugs when renal biopsy is not possible. Additional studies are needed to explore the mechanisms behind these findings, and greater numbers of cases with outcome data are needed to determine if there are truly significant differences in survival times in dogs with different forms of ICGN.

CHAPTER IV

CORRELATION OF ELECTROPHORETIC URINE PROTEIN BANDING
PATTERNS WITH SEVERITY OF RENAL DAMAGE AND SURVIVAL IN DOGS
WITH PROTEINURIC CHRONIC KIDNEY DISEASE

Introduction

Chronic kidney disease (CKD) is a common and progressive disease in dogs that can result in significant morbidity and mortality.^{18,124,159,160} Not only is the onset of CKD insidious, but current non-invasive methods for evaluating CKD in dogs lack the ability to accurately assess severity of glomerular and tubulointerstitial (TI) damage, which must be characterized by histologic evaluation of a renal biopsy.^{14,15,18,77,124} While renal histology allows diagnosis of the cause of kidney disease and assessment of the severity of glomerular and TI damage, performing a renal biopsy is often not practical for patients due to financial constraints, anesthetic requirements, and other risk factors related to surgery. Additionally, biopsy findings do not always correspond with kidney function, particularly with regard to lesions that can be patchy in nature, such as TI fibrosis. Thus, minimally invasive, inexpensive methods for determining the degree of renal damage without the need for biopsy might be an attractive option for clinical veterinarians.

Urine of healthy dogs contains little to no protein because of the selective permeability of the glomerulus and the protein reabsorption capacity of the proximal tubules.^{14,15} In health, the glomerular filtration barrier allows free passage of low

molecular weight (LMW) proteins (< 40 kilodaltons (kDa)) but restricts the passage of most intermediate molecular weight (IMW) proteins (≥ 40 kDa and < 70 kDa) and nearly all high molecular weight (HMW) proteins (≥ 70 kDa).¹⁵

Renal proteinuria, or the abnormal presence of proteins in the urine due to renal disease, is common in canine CKD.^{14,124,160} Major mechanisms of renal proteinuria include: the abnormal transglomerular passage of proteins due to increased permeability of the glomerular capillary wall, the impaired reabsorption of proteins by proximal tubular epithelial cells, and the release of proteins and enzymes from damaged tubular epithelial cells.^{12,15,37,77,126} In addition to being a hallmark sign of kidney disease in dogs, renal proteinuria is also associated with kidney disease progression.^{15,18,126} For example, a UPC ≥ 1.0 in dogs with chronic renal failure was significantly correlated with faster progression to end stage renal failure compared to dogs with a UPC < 1.0 .¹²⁶ Several other studies have shown that the role of proteinuria in CKD and progression to end stage renal failure is often underappreciated, and proteinuria might be overlooked as an early biomarker of kidney disease.^{18,124,126,160,161}

Gel electrophoresis by various methodologies (e.g. sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and sodium dodecyl sulfate agarose gel electrophoresis (SDS-AGE)) has been used to evaluate patterns of proteinuria in humans, rats, and dogs with various nephropathies, including correlation of electrophoretic protein banding patterns with renal histology and renal function.^{19-26,28,162-165} In all species, evaluation of urine protein banding patterns by gel electrophoresis has been shown to be a useful clinical tool to determine the site of kidney

damage (i.e., glomerular versus tubular versus both).^{19-26,28,162-166} An abundance of HMW proteins and IMW proteins indicates glomerular proteinuria while an abundance of LMW proteins signifies tubular proteinuria.^{20,21,25,28,163-165} Studies in dogs have generally agreed that urine protein banding patterns correlate well with histologic renal lesions, and protein banding patterns consistent with predominantly glomerular, predominantly tubular, and mixed glomerular and tubular damage have been established.^{20,21,23,26} In fact, several LMW proteins (12, 15, and 21 kDa) were found to be significantly associated with the degree of TI damage severity, and presence of 12 or 15 kDa protein bands was highly specific for severe TI damage.²⁶ Limited studies have explored the sensitivity and specificity of electrophoretic methods for detection of glomerular and TI damage²⁶ or proteinuria^{19,20,167} in dogs. Good sensitivity but poor specificity was found for the ability of SDS-AGE to detect glomerular and TI lesions as compared to histology,²⁶ while good sensitivity but variable specificity has been found for the ability of SDS-AGE to detect borderline proteinuria or proteinuria as compared to UPC measurements¹⁹ or quantification of urinary albumin¹⁶⁷ or immunoglobulin G.²⁰

The first major objective of this study was to determine the ability and accuracy of urine electrophoretic protein banding patterns to identify the presence and degree of glomerular and TI damage as compared to renal histology and widely available noninvasive biomarkers in dogs with naturally occurring proteinuric CKD. A second objective was to determine whether urine protein banding patterns were associated with risk of death due to renal disease in the same cohort of dogs.

Materials and Methods

Sample Collection and Processing

Dogs with Proteinuric CKD

Urine supernatant and kidney biopsy and necropsy tissues from dogs with clinician determined renal disease were submitted to the International Veterinary Renal Pathology Service (IVRPS) between January 2008 and September 2014. All samples were shipped chilled and were received and processed within 24 hours post-collection. Urine specific gravity (USG)^s, urine protein:creatinine (UPC)^{t,u,v}, and serum creatinine (sCr)^{t,u} were determined in our laboratory as previously described,¹² and urine supernatant from each dog was aliquoted and stored at -80°C. Cases with an active sediment (hematuria, pyuria/bacteriuria, or both) on the submitted sample or within 4 weeks of kidney biopsy were excluded from analysis. Hematuria was based on gross discoloration or > 5 red blood cells per 40x field on sediment examination. Pyuria was based on > 5 white blood cells per 40x field on sediment examination. Bacteriuria was present if bacteria were seen on sediment examination or if there was a recent positive culture result. Kidney biopsies were processed for light (LM) and transmission electron microscopy (TEM) as previously described.²³ Renal disease was diagnosed if the dog had persistent proteinuria, azotemia, or both, and dogs were determined to have CKD (3+ months of evidence of renal disease and/or chronic renal changes on abdominal

^s Rhino VET360 Veterinary Clinical Refractometer, Reichert Technologies, Depew, NY

^t Creatinine LiquiColor Test (Endpoint), Stanbio Laboratory, Boerne, TX

^u Sirrus Clinical Chemistry Analyzer, Stanbio Laboratory, Boerne, TX

^v Protein, Micro LiquiColor Test (CSF and Urine), Stanbio Laboratory, Boerne, TX

ultrasound or renal histology, such as fibrosis), acute kidney injury (AKI), AKI superimposed on CKD, or insufficient information available to determine renal disease chronicity.

Clinically Healthy Dogs

Urine was collected from client-owned dogs that were deemed healthy based on history, physical examination, complete blood count, serum chemistry, urinalysis, and UPC. Additionally, urine supernatant and kidney biopsy tissue previously collected from healthy control dogs from studies of dogs with early onset CKD due to X-linked hereditary nephropathy (XLHN) were used.¹⁵⁴ Urine was collected by cystocentesis or free-catch, and urinalysis was performed within 2 hours of collection. Cases were excluded from analysis if hematuria, pyuria/bacteriuria, or both were identified. UPC and sCr were determined for each sample as previously described.¹² Urine supernatant and serum were stored at -80°C prior to analysis. Samples collected from client-owned dogs were obtained with client consent.

Histologic Analysis and Scoring of Kidney Biopsies

Kidney biopsies were evaluated for glomerular and TI damage and scored as previously described.¹² The score for glomerular damage based on LM used a 0 – 3 ordinal scale: 0 = normal glomeruli; 1 = mild or focal lesions; 2 = moderate lesions; and 3 = severe lesions. The glomerular damage score based on TEM also used a 0 – 3 ordinal scale: 0 = normal ultrastructure; 1 = mild reversible ultrastructural lesions; 2 = moderate

irreversible ultrastructural lesions; and 3 = severe irreversible ultrastructural lesions. TI damage scores were determined for different components of damage including TI fibrosis (score range: 0 – 5), tubular atrophy (score range: 0 – 3), tubular degeneration/necrosis/regeneration (score range: 0 – 5), and TI chronic inflammation (score range: 0 – 5) with 0 indicating normal TI/no lesions for each component and 3 or 5 indicating severe lesions.

Bis-Tris Gel Electrophoresis of Urine Supernatant

Non-reducing, denaturing gel electrophoresis was performed on all canine urine samples, typically within 2 months of collection, using precast 12-well, 4 – 12% Bis-Tris gels^w in the Invitrogen XCell Sure Lock Mini-Cell Electrophoresis system.^x Urine samples were diluted with ultrapure water based on USG to a volume of 30 µl, followed by addition of 10 µl lithium dodecyl sulfate (LDS) sample buffer^y for a final volume of 40µl. The urine volume component was calculated as: urine volume (µl) = 0.065/(USG-1). This formula ensured that for highly concentrated urine samples, a minimum of 1 µl of urine was used for dilution. Samples were heated at 70°C for 10 minutes, and 20 µl of each sample was loaded into a lane of the precast gel, leaving the first and last lanes for the molecular weight standard^z (5 µl). Each sample and standard was loaded onto duplicate gels, which were run according to manufacturer protocol (200V for 35

^w NuPAGE 4-12% Bis-Tris Gel, Life Technology Corporation, Carlsbad CA

^x Invitrogen XCell SureLock Mini-Cell Electrophoresis System, Invitrogen Life Technologies, Carlsbad CA

^y NuPAGE LDS Sample Buffer [4X], Invitrogen Life Technologies, Carlsbad CA

^z Mark12 Unstained Standard, Invitrogen Life Technologies, Carlsbad CA

minutes) in 2-(*N*-morpholino)ethanesulfonic acid (MES) sodium dodecyl sulfate (SDS) buffer^{aa}. Following water washes, gels were stained with Imperial Protein Stain^{bb} for 2 hours and destained overnight with ultrapure water. The following day, gels were digitally photographed^{cc} and dried by soaking them in a drying solution (prepared as 20 mL glycerol, 200 mL 100% ethanol, and 780 mL ultrapure water) for 20 minutes. Gels were then sandwiched between transparent cellulose sheets.

To determine the repeatability of the gel electrophoresis procedure and the protein banding patterns produced, 10 samples were randomly selected from possible candidates to represent the range of banding patterns observed from dogs with naturally occurring CKD. Samples were run on three different days, randomizing sample lane location for each run. Each aliquot used for each run had the same number of freeze-thaw cycles.

Computer and Visual Analysis of Bis-Tris Gels

Digital photographs of each gel were analyzed using a commercially available software for gel fingerprint analysis.^{dd} Standard instructions provided in the software manual were followed to remove the background of the digital gel image.

A reference system was established to assign the molecular weight of the standards, and the protein bands for each dog's urine sample were normalized to this

^{aa} NuPAGE MES SDS Running Buffer [20X], Invitrogen Life Technologies, Carlsbad, CA

^{bb} Imperial Protein Stain, ThermoFisher Scientific, Rockford, IL

^{cc} Gel Doc XR + System, Bio-Rad Laboratories Inc., Hercules, CA

^{dd} BioNumerics Version 6.6, Applied Maths, Austin, TX

reference system. Using default settings to limit manual correction of the bands, the software program automatically searched for protein bands.

Following the software's automatic assignment of bands for each sample, the dried gels were used to verify the presence of bands compared to the digital image. Each band was verified by at least two researchers.

The total number and molecular weights of protein bands for each sample were exported to Microsoft Excel. For the repeatability study, the inter-run percent coefficient of variation (CV%) was calculated for the total number of bands identified for each sample.

Gel Score Analysis

Digital images of gels and dried gels from all cases used in this study were visually reviewed by 3 of the authors (SL, JAH, MBN) with previous experience in the analysis of glomerular and tubular protein banding patterns. A scoring algorithm was developed for the glomerular (IMW and HMW) and tubular (LMW) banding patterns. Glomerular severity scores ranged from 0 – 3: 0 – normal/no glomerular proteinuria; 1 – mild glomerular proteinuria; 2 – moderate glomerular proteinuria; 3 – severe glomerular proteinuria. Tubular severity scores ranged from 0 – 4: 0 – normal/no tubular proteinuria; 1 – minimal tubular proteinuria; 2 – mild tubular proteinuria; 3 – moderate tubular proteinuria; 4 – severe tubular proteinuria. Tables 9 and 10 detail the criteria used to assign the glomerular and tubular gel scores, and Figure 18 demonstrates application of the gel scores for a set of samples with varying degrees of proteinuria.

Table 9. Glomerular gel scoring system for dogs.

Glomerular Gel Score	Gel Findings		Figure 18 Example
	Albumin band characteristics	Other band features	
0 (Normal)	Faint and smaller/less prominent than the corresponding MW standard (~55 kDa) +/- 1-2 additional faint bands in the same region.	+/- 1 faint band at ~ 88-101 kDa (Tamm-Horsfall protein).	Lane 2 & 9
1 (Mild)	At least the size of the corresponding MW standard and ranges up to 3 times the size of the corresponding MW standard.	<10 HMW bands; 1 – 2 bands at ~ 130-150 kDa may be prominent but thin (about the same size/intensity as a MW standard).	Lanes 3 & 4
2 (Moderate)	a) Prominent, dark staining, and extends \leq 20% of the distance between the 55.4 kDa and 36.5 kDa MW standards. OR b) Extends \geq 25% of the distance between the 55.4 kDa and 36.5 kDa MW standards.	If option a) from albumin features: AND $>$ 1 band \geq 200 kDa. If option b) from albumin features: AND less than 5 bands \geq 200 kDa +/- up to 2 dark bands at ~ 130-150 kDa that approach the darkness/intensity of the albumin band.	Lanes 5 & 6
3 (Severe)	Extends \geq 25% of the distance between the 55.4 kDa and 36.5 kDa MW standards.	\geq 5 bands \geq 200 kDa and/or \geq 3 dark bands at ~ 130-150 kDa that approach the darkness/intensity of the albumin band.	Lanes 7 & 8

HMW, high molecular weight; kDa, kilodaltons.

Table 10. Tubular gel scoring system for dogs.

Tubular Gel Score	Gel Findings		
	No or minimal glomerular proteinuria	Glomerular proteinuria present	Figure 18 Example
0 (Normal)	No LMW bands should be present, unless the dog is an intact male, in which case 3 variably prominent prostatic fluid protein bands may be present at ~ 8 – 9.5 kDa, 15 – 16 kDa, and 26 – 27 kDa.	A faint band might be present at ~ 24-25.5 kDa in addition to the prostatic fluid protein bands.	Lane 2
1 (Minimal)	1 – 2 faint LMW bands (excluding prostatic fluid protein bands).	≤ 5 faint LMW bands (excluding the 24-25.5 kDa and prostatic fluid protein bands)	Not shown
2 (Mild)	3 – 9 LMW bands, with 0 – 1 dark/intense bands (excluding prostatic fluid protein bands).	<p>≤ 5 LMW bands with exactly 1 dark/intense band (excluding the 24-25.5 kDa and prostatic fluid protein bands).</p> <p>OR</p> <p>≥ 6 but < 10 LMW bands with 0 to 1 dark/intense bands (excluding the 24-25.5 kDa band and prostatic fluid protein bands).</p> <hr/> <p>- If there is more than 1 band below 24-25.5 kDa (excluding prostatic fluid bands), the score should be at least mild.</p>	Lanes 3-5
3 (Moderate)	≥ 10 but < 13 LMW bands (excluding prostatic fluid protein bands), with ≤ 2 dark/intense LMW bands.	< 13 LMW bands with 2 dark/intense bands (excluding the 24-25.5 kDa and prostatic fluid protein bands).	Lanes 6 & 9
4 (Severe)	<p>≥ 13 LMW bands (excluding prostatic fluid protein bands).</p> <p>AND/OR</p> <p>≥ 3 dark/intense LMW bands.</p>	<p>≥ 13 LMW bands (excluding the 24-25.5 kDa and prostatic fluid protein bands).</p> <p>AND/OR</p> <p>≥ 3 dark/intense LMW bands.</p>	Lanes 7 & 8

LMW, low molecular weight; kDa, kilodaltons.

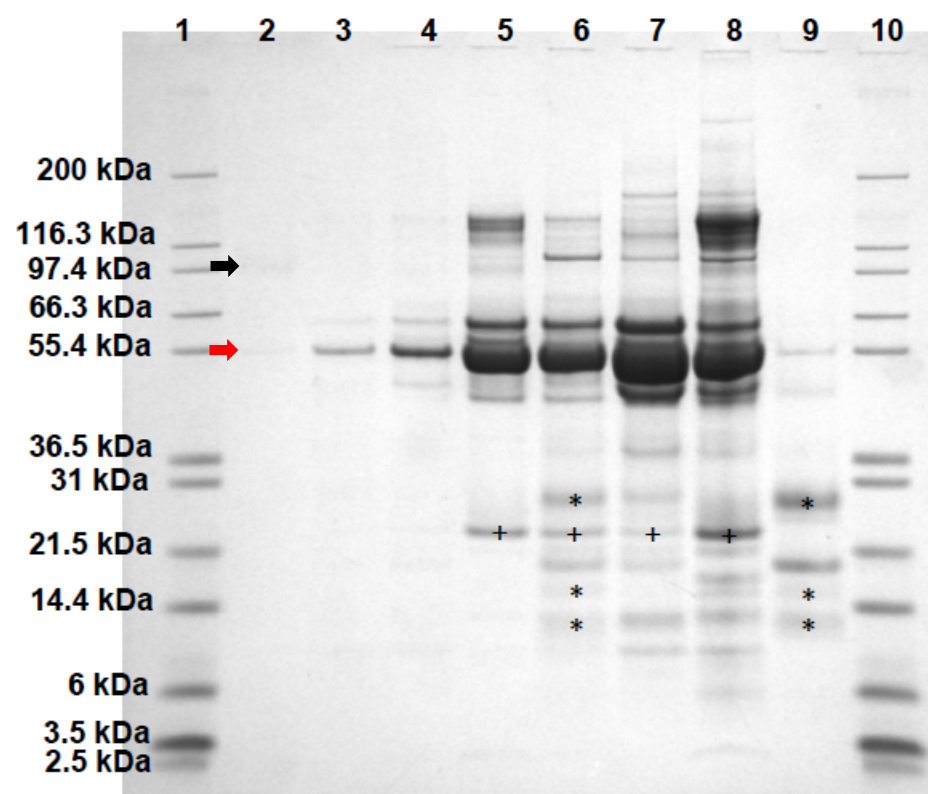


Figure 18. Representative urine samples from dogs with a wide spectrum of proteinuria resolved using gel electrophoresis to demonstrate application of the gel scoring system.

Lanes 1 and 10: molecular weight standard.

Lane 2: Urine sample from a healthy, non-proteinuric dog. Glomerular and tubular scores = 0.

Lanes 3 – 9 represent urine samples from proteinuric dogs where glomerular proteinuria predominates.

Lane 3: Glomerular score = 1; Tubular score = 2.

Lane 4: Glomerular score = 1; Tubular score = 2.

Lane 5: Glomerular score = 2; Tubular score = 2.

Lane 6: Urine sample from an intact male dog with proteinuria. Glomerular score = 2; Tubular score = 3.

Lane 7: Glomerular score = 3 (although faint, there are 5 bands ≥ 200 kDa); Tubular score = 4.

Lane 8: Glomerular score = 3; Tubular score = 4.

Lane 9: Urine sample from an intact male dog with proteinuria. Glomerular score = 0 (although the albumin band is slightly darker than typically expected for a normal dog, it is faint and likely secondary to tubular damage); Tubular score = 3.

* denotes prostatic fluid proteins.

+ denotes 24-25.5 kDa protein band.

Black arrow denotes Tamm-Horsfall Protein band.

Red arrow denotes albumin band.

kDa, kilodalton; HMW, high molecular weight; LMW, low molecular weight.

Survival Data

Primary and referring veterinarians and owners were contacted between 2 months to up to 8 years post-biopsy at multiple intervals to obtain information regarding the dog's health status following renal biopsy. At the point when the dog was deceased, the amount of time to death post-biopsy, type of death (spontaneous versus humane euthanasia), and cause of death or decision to euthanize (e.g., renal disease progression) were recorded.

Statistical Analysis

Prediction of Glomerular and Tubular Damage

Sensitivities, specificities, and accuracy were calculated for the ability of glomerular gel scores, $\text{UPC} \geq 0.5$, $\text{UPC} \geq 1$, and $\text{UPC} \geq 2$ to predict the presence of glomerular damage when either an LM or TEM glomerular damage score > 0 was set as the gold standard for presence of glomerular damage. Sensitivities, specificities, and accuracy were also calculated for the ability of tubular gel scores, $\text{SCr} \geq 1.4 \text{ mg/dL}$, and $\text{SCr} \geq 1.4 \text{ mg/dL} + \text{USG} < 1.035$ to predict the presence of TI damage when a score > 0 for any of the individual TI damage scores (fibrosis, atrophy, degeneration, chronic inflammation) was set as the gold standard for presence of TI damage. Accuracy was calculated as: (number of true positives + number of true negatives) / (total number of tests).

Gel Score and Biomarker Correlations

To determine if the gel scores were correlated with the biopsy damage scores, pairwise polychoric correlation analysis was used to estimate the correlation between glomerular gel scores and LM and TEM glomerular damage scores and between tubular gel scores and the individual TI damage scores. Additionally, pairwise polychoric correlation analysis was used to estimate the correlation between the LM and TEM glomerular damage scores. Polychoric correlation analysis estimates the correlation between 2 theorized normally distributed continuous latent variables from two observed ordinal variables.¹⁶⁸ Principal component analysis was performed on all of the individual TI biopsy scores to generate a composite score, and polychoric correlation analysis was performed between the tubular gel score and the composite TI biopsy score. Finally, to investigate the overall association between a single composite gel score and a single composite biopsy score, canonical correlation analysis was performed based on the polychoric correlation matrix. Canonical correlation analysis is a multivariate statistical analysis method that assesses the correlation between two sets of data.¹⁶⁹ Correlation strength for all analyses was defined as: weak: $r = 0.0 - 0.39$; moderate: $r = 0.4 - 0.69$; or strong: $r = 0.7 - 1.0$.

Survival Analysis

For the glomerular and tubular gel scores, LM and TEM glomerular biopsy scores and the TI biopsy scores, UPC, sCr, and presence of LMW bands at approximately 21 kDa (range: 20.73-21.66 kDa), 15 kDa (range: 15.11-15.77 kDa), 12

kDa (range: 12.07-12.78 kDa), and 10 kDa (range: 9.96-10.60 kDa), Cox proportional hazard regression models (accounting for the biomarker or score and age as covariates) were used to evaluate the relationship between each variable and the hazard ratio (HR) or relative risk of death due to renal disease post-biopsy using all follow-up data obtained (n = 117 dogs). For variables with significant HRs, the median time to death post-biopsy due to renal disease was determined for different cutoff values of each variable.

The ability of gel scores to predict glomerular and TI damage and risk of death/survival time analysis was performed using Stata version 11^{ee}, setting $P < 0.05$. Gel score and biomarker correlations were performed in R version 3.2.2 using the polycor package 0.7-8^{ff}.

Results

CKD Dogs

Urine supernatant and kidney biopsy and necropsy tissue were submitted and initially analyzed from 254 dogs with renal disease. Urinalyses were performed on the submitted urine samples by the referring veterinarians or on a sample within 4 weeks of biopsy collection in 71% of these cases, of which 30 dogs (16.7%) had hematuria, 4 dogs had pyuria/bacteriuria (2.22%), and 10 dogs had both hematuria and pyuria/bacteriuria (5.55%). These 44 dogs were excluded from the study leaving 210

^{ee} Stata Version 11, Stata Corp. LP, College Station, TX

^{ff} R version 3.2.2; Polycor package version 0.7-8

dogs for further analysis. Of the 210 remaining dogs, 92 (43.81%) were spayed females, 70 (33.33%) were neutered males, 29 (13.81%) were intact males, and 19 (9.05%) were intact females. While a variety of breeds were represented, Labrador Retrievers/Labrador Retriever-mixes were the most common (11.92%). Additionally, a broad range of ages (2 months-14 years) was represented, and the median age was 6.5 years.

Outcome/prognostic information was collected post-biopsy for 117 (56%) of the dogs included in the analysis. Fifty-eight dogs died or were euthanized due to renal-related causes with a median time to death following biopsy (excluding 6 necropsy samples) of 183 days (range: 2 – 1,460 days).

Kidney disease was determined to be present based on persistent proteinuria in 102 dogs (48.6%), azotemia in 22 dogs (10.5%), and both proteinuria and azotemia in 86 dogs (41.0%). Based on known timeframe of disease symptoms and/or presence of chronicity on ultrasound or renal histology, CKD was confirmed for 189 (90%) dogs. Of the remaining 21 dogs, 8 had evidence of AKI either alone (3 dogs) or concurrently with CKD (5 dogs) while 13 were of unknown duration.

Healthy Dogs

Urine supernatant was collected from 18 healthy client-owned dogs, and urine supernatant and kidney biopsy tissue was used from 3 healthy control dogs from the XLHN colony. Of these 21 control dogs, 10 dogs (48%) were excluded based on hematologic, biochemical, or urinalysis results outside of the laboratory reference ranges, leaving 11 control dogs for further analysis.

Of the 11 remaining control dogs, there were 4 (36.36%) spayed females, 3 (27.27%) neutered males, and 4 (36.36%) intact males. The control dogs were composed of the following breeds: 6 (54.55%) Labrador Retriever/ Labrador Retriever mixes; 2 (18.18%) Border Collie/ Border Collie mixes; 1 (9.09%) German Shepherd; 1 (9.09%) Catahoula Leopard dog mix; and 1 (9.09%) Golden Retriever mix. The median age of the control dogs was 5 years (range: 9 months-10 years).

Histopathologic Findings and Scores

The number of cases assigned glomerular and TI biopsy damage scores was less than the entire set of dogs in the study either due to limited quality of biopsy tissue that could be used for scoring or because a histologic diagnosis was achieved based on LM and continued analysis by TEM was cancelled by the referring veterinarian. Most dogs in the study had at least moderate glomerular damage (score ≥ 2) on both LM and TEM (Table 11). In contrast, TI damage was more limited in these samples, with most cases receiving damage scores < 2 (normal to mild damage), with few cases receiving a score ≥ 3 (Table 11).

Gel Electrophoresis

Gels from the 11 clinically normal, non-proteinuric (control) dogs consistently displayed a minimal/faint protein band at approximately 55 kDa, in line with the albumin standard¹⁹⁻²⁶ and a faint band at approximately 100 kDa, consistent with Tamm-Horsfall protein.²² All female control dogs and neutered male control dogs displayed no

bands in the tubular region. Intact male control dogs displayed 3 slightly prominent protein bands in the tubular region at 26-27 kDa, 15-16 kDa, and 8-9.5 kDa, likely consistent with prostatic fluid protein bands^{20,30,170-172} similar to those in Figure 18.

Gels from the CKD dogs displayed a variety of glomerular and tubular urine protein banding patterns, with numbers and intensities of bands varying dramatically. Using the developed scoring criteria (Tables 9 and 10), the most frequent glomerular gel score was moderate (score = 2), which was similar to the biopsy scores. In contrast to the biopsy scores, most tubular gel scores were mild and moderate (score = 2 and 3) (Table 11). Figure 19 demonstrates that cases with primary glomerular disease had median glomerular gel scores and LM and TEM glomerular biopsy scores that were higher than cases with primary tubular disease. Medians for averaged TI biopsy scores (averages of the fibrosis, atrophy, degeneration, and chronic inflammation scores) were slightly lower in cases with primary glomerular versus primary tubular disease, while median tubular gel scores were actually higher in cases with primary glomerular versus tubular disease.

To determine repeatability of the gel electrophoresis method, each of 3 gel runs was analyzed both by determining the total number of protein bands detected in each sample as well as visual scoring for glomerular and tubular damage. For the total number of bands present between runs for individual samples, a CV% of < 10% was achieved for all but one sample; the remaining sample had a CV% of 11.2%. Based on visual examination, no major differences in the number or intensity of bands were seen among the repetitions. Additionally, using the developed gel scoring system (Tables 9 and 10), each sample received the same glomerular gel score and all but one received the same

tubular gel score at each repeat. For one run of this individual sample, a slightly higher tubular gel score was assigned; however, this likely reflects differences in subjective scoring rather than true differences in banding patterns, band numbers, and/or band intensity.

Sensitivity and Specificity of Gel Scores for Detection of Glomerular and TI Damage

Using presence of glomerular damage on LM or TEM as the gold standard, sensitivities and specificities of a glomerular gel score ≥ 1 and a UPC ≥ 0.5 , ≥ 1 , and ≥ 2 were determined (Table 12). Greatest sensitivity was seen with a glomerular gel score ≥ 1 (96.43%), while all analyses had 100% specificity for glomerular damage. Similarly, histologic TI damage was used to determine sensitivities and specificities of a tubular gel score ≥ 1 , sCr ≥ 1.4 mg/dl, and combined sCr ≥ 1.4 mg/dl and USG ≤ 1.035 (Table 13). All analyses had 100% specificity for presence of tubular damage, but highest sensitivity was observed with a tubular gel score ≥ 1 (89.34%) whereas sensitivities for sCr ≥ 1.4 mg/dl and combined sCr ≥ 1.4 mg/dl and USG ≤ 1.035 were poor.

Table 11. Numbers/frequency of cases in each gel score and biopsy score category. Gel scores and glomerular biopsy scores followed an ordinal scoring system. Tubular/tubulointerstitial biopsy scores followed a continuous scoring system.

	Score 0	Score 1	Score 2	Score 3	Score 4	
Glomerular Gel Score N = 221	24 (10.9%)	22 (10.0%)	101 (45.7%)	74 (33.5%)	N/A	
Tubular Gel Score N = 221	32 (14.5%)	32 (14.5%)	70 (31.7%)	60 (27.2%)	27 (12.2%)	
Glomerular Biopsy Score: Light Microscopy N = 201	18 (9.0%)	53 (26.4%)	83 (41.3%)	47 (23.4%)	N/A	
Glomerular Biopsy Score: Transmission Electron Microscopy N = 171	4 (2.3%)	41 (24.0%)	77 (45.0%)	49 (28.7%)	N/A	
	Score 0-<1	Score 1-<2	Score 2-<3	Score 3-<4	Score 4-<5	Score ≥5
Tubulointerstitial Fibrosis Biopsy Score N = 200	103 (51.5%)	46 (23.0%)	30 (15.0%)	15 (7.5%)	5 (2.5%)	1 (0.5%)
Tubular Atrophy Biopsy Score N = 200	175 (87.5%)	21 (10.5%)	4 (2.0%)	N/A	N/A	N/A
Tubular Degeneration Biopsy Score N = 197	98 (49.8%)	56 (28.4%)	31 (15.7%)	12 (6.1%)	N/A	N/A
Tubulointerstitial Chronic Inflammation Biopsy Score N = 199	123 (61.8%)	65 (33.7%)	11 (5.5%)	N/A	N/A	N/A

N/A, not applicable.

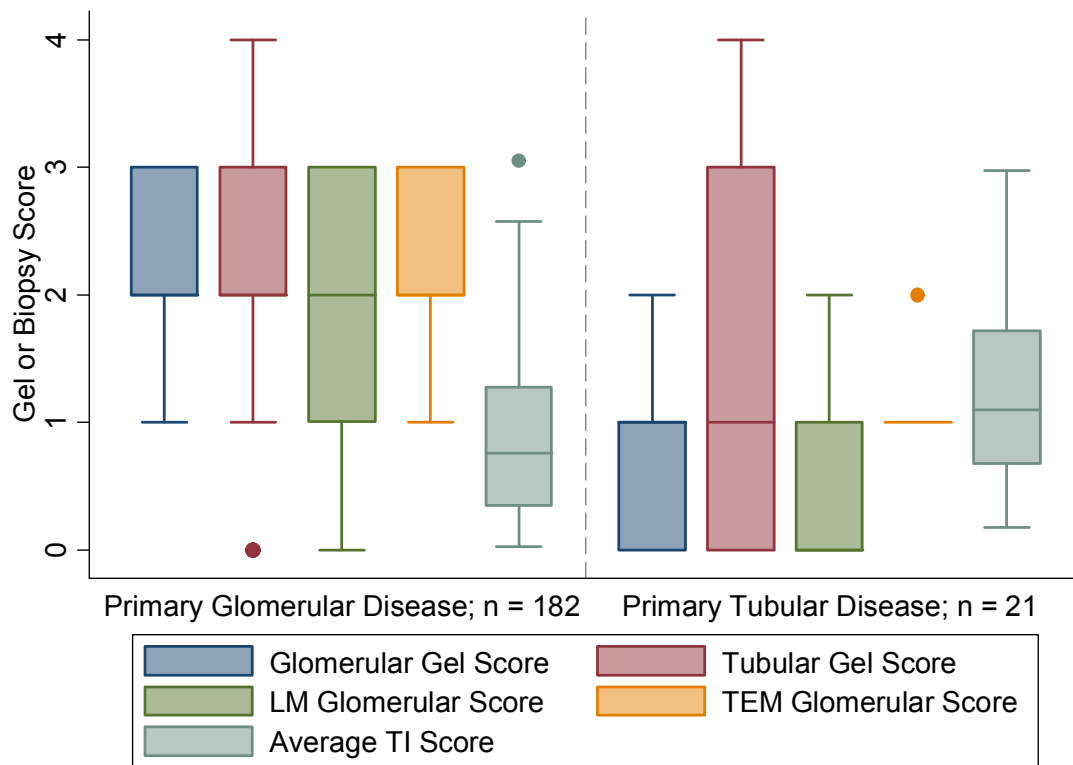


Figure 19. Boxplots representing the range of gel and biopsy scores, with cases categorized as primary glomerular or primary tubular disease. LM, light microscopy; TEM, transmission electron microscopy; TI, tubulointerstitial. Average TI score represents the average of the 4 component scores for TI damage (fibrosis, atrophy, degeneration, and chronic inflammation)

Table 12. Ability of glomerular gel scores ≥ 1 and UPC ≥ 0.5 , 1, and 2 to predict glomerular damage based on light and electron microscopic analysis of renal biopsies.

	Sensitivity	Specificity	Accuracy	PPV	NPV
Glomerular Gel Score ≥ 1	96.43%	100.00%	96.47%	100.00%	25.00%
UPC ≥ 0.5	92.61%	100.00%	92.70%	100.00%	13.00%
UPC ≥ 1	88.64%	100.00%	88.76%	100.00%	9.5%
UPC ≥ 2	80.11%	100.00%	88.76%	100.00%	5.7%

UPC, urine protein:creatinine; PPV, positive predictive value; NPV, negative predictive value.

Table 13. Ability of tubular gel scores ≥ 1 , sCr ≥ 1.4 mg/dl, and combined sCr ≥ 1.4 mg/dl and USG ≤ 1.035 to predict tubulointerstitial damage based on light microscopic analysis of renal biopsies.

	Sensitivity	Specificity	Accuracy	PPV	NPV
Tubular Gel Score ≥ 1	89.34%	100.00%	89.50%	100.00%	5.00%
sCr ≥ 1.4 mg/dl and USG ≤ 1.035	51.49%	100.00%	52.20%	100.00%	1.3%
sCr ≥ 1.4 mg/dl	58.91%	100.00%	59.51%	100.00%	1.2%

sCr, serum creatinine; USG, urine specific gravity; PPV, positive predictive value; NPV, negative predictive value.

Correlations of Gel Scores with Severity of Glomerular and TI Damage

Figure 20 displays the r-values for the pairwise polychoric correlations between pairs of gel and biopsy scores. The highest r-values were found for the correlation between the glomerular gel score and the TEM glomerular biopsy score ($r = 0.68$), followed by the correlations between the LM and TEM glomerular biopsy scores ($r = 0.64$) and between glomerular and tubular gel scores ($r = 0.57$). The glomerular gel score was less correlated with the LM glomerular biopsy score ($r = 0.43$), and the tubular gel score correlated only weakly to moderately with the different TI biopsy scores. The polychoric correlation between the tubular gel score and the composite TI biopsy score

was also only moderate ($r = 0.42$). Standard errors for all polychoric correlations ranged from 0.05 for the highest correlations to 0.09 for the lowest correlations (data not shown). The canonical correlation analysis between the composite gel and biopsy scores found a strong correlation ($r = 0.73$), including between both glomerular and tubular damage scores within the same case (data not shown). Further analysis of the canonical loading vector coefficients revealed that this composite correlation was primarily due to the correlation between the glomerular gel scores and TEM biopsy scores, confirming that these two scores play major parts in capturing the overall association between the gel scores and the biopsy scores.

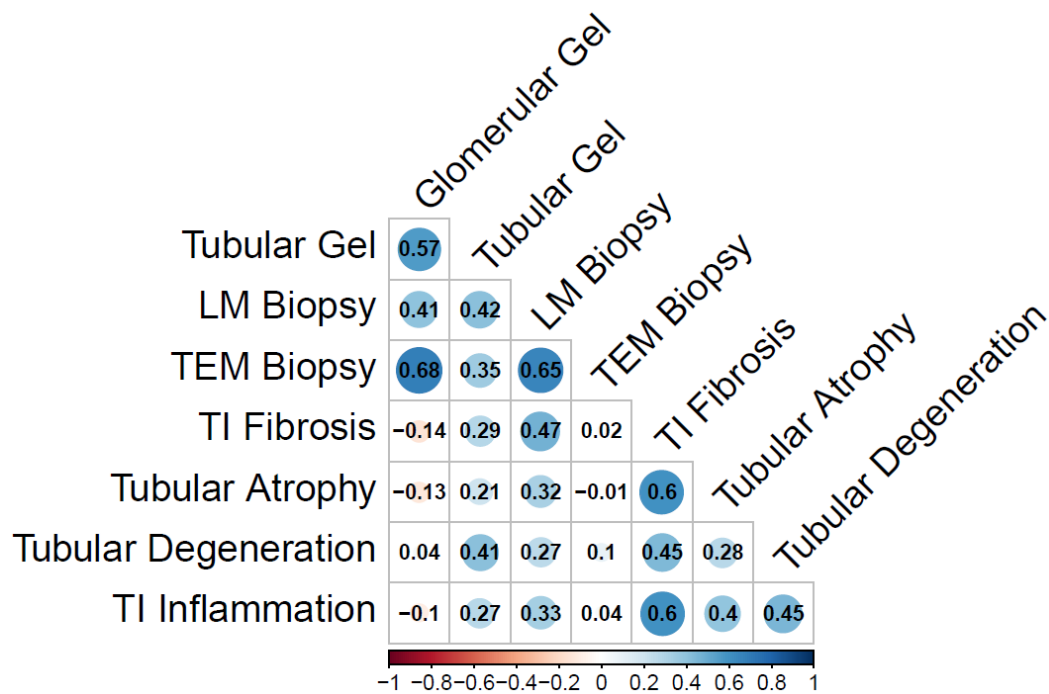


Figure 20. Pairwise polychoric correlation analysis between each of the gel and biopsy scores. LM, light microscopy; TEM, transmission electron microscopy; TI, tubulointerstitial. The X-axis indicates the strength and sign of the correlations. White colors indicate lack of correlation. Light to dark red colors indicate weak to strong negative correlations. Light to dark blue indicate weak to strong positive correlations.

Survival Analysis

Cox proportional hazard models were generated to determine if glomerular and tubular gel scores, presence of tubular bands at 21, 15, 12, and 10 kDa, LM and TEM glomerular biopsy scores and individual TI biopsy scores, and UPC and sCr were associated with an increased risk of death occurring due to renal disease post-biopsy. LM glomerular biopsy score, tubular gel score, TI fibrosis score, tubular degeneration score, presence of bands at 10 and 15 kDa, sCr, and UPC were all significantly associated with an increased risk (increased HR) of death due to renal disease when age was included as a covariate (Table 14). Presence of a band at 10 kDa had the highest HR of 2.70, translating to a 2.7:1 odds of a shorter survival time in dogs with a 10 kDa band versus those without it. Similarly, presence of a LMW band at 15 kDa had an HR of 1.96 and the tubular gel score had an HR of 1.65, and thus the odds of a shorter survival time in each situation is 1.96:1 and 1.65:1. Of note, for most of the clinical variables, age was a significant covariate when determining relative risk of death due to renal disease. Using the Cox survival models, figures 21-24 illustrate the probability of survival for a dog of median age at different starting values of the biomarkers and biopsy/gel scores that had a P-value < 0.01 (including the glomerular LM biopsy score, tubular gel score, tubular fibrosis score, and sCr).

For each of the variables with significant HRs from the Cox proportional hazard models, meaningful cutoff values were determined and the median number of days to death due to renal disease post-biopsy was calculated for these cutoff values (Table 15). For example, an LM glomerular biopsy score of 0-1 had a median time to death of 634

days, while an LM glomerular biopsy score of 2-3 had a median time to death of 139 days. All biomarkers and scores had a longer time to death for the “lower” cutoff category, except for UPC below 2.0, which had a slightly shorter median time to death than a $UPC \geq 2.0$.

Table 14. Relative risk (hazard ratios) of death due to renal disease in dogs using multivariate Cox survival models for selected biomarkers and biopsy or gel scores including age as a covariate. The unit of increase for each biomarker or score and age is noted in parentheses in the first column. Each row depicts a separate survival model that includes 2 covariables (biomarker or score and age).

	N	HR for Score/Biomarker		HR for Age	
Biomarker or Biopsy/Gel Score + Age (1 year)		HR (95% CI)	P-value	HR (95% CI)	P-value
Glomerular LM biopsy score (1 score point)	94	1.58 (1.13-2.21)	0.008	1.09 (1.00-1.20)	0.053
Tubular gel score (1 score point)	98	1.65 (1.29-2.11)	0.000	1.10 (1.00-1.20)	0.042
TI fibrosis score (1 score point)	94	1.53 (1.17-2.01)	0.002	1.12 (1.03-1.23)	0.011
Tubular degeneration score (1 score point)	94	1.44 (1.05-1.98)	0.023	1.09 (1.00-1.19)	0.056
Presence of band at 10 kDa	98	2.70 (1.18-6.17)	0.019	1.11 (1.01-1.22)	0.024
Presence of band at 15 kDa	98	1.96 (1.10-3.49)	0.023	1.11 (1.02-1.22)	0.022
UPC (1 unit)	98	1.05 (1.01-1.09)	0.015	1.11 (1.01-1.21)	0.035
sCr (1 mg/dL)	96	1.37 (1.23-1.52)	0.000	1.13 (1.03-1.25)	0.011

HR, hazard ratio; CI, confidence interval; LM, light microscopy; TI, tubulointerstitial; kDa, kilodalton; UPC, urine protein:creatinine; sCr, serum creatinine.

Table 15. Median days to death due to renal disease post-biopsy for selected scenarios as based on significant findings from Cox survival models.

Scenario	N	Days to Death due to Renal Disease Median (Range)
Glomerular LM biopsy score: 0-1	11	634 (3-1460)
Glomerular LM biopsy score: 2-3	41	139 (2-1190)
Tubular gel score: 0-2	21	259 (3-1460)
Tubular gel score: 3-4	31	122 (2-1190)
TI fibrosis score: < 3	43	244 (2-1460)
TI fibrosis score: ≥ 3	9	22 (3-758)
Tubular degeneration score: < 2	37	259 (2-1460)
Tubular degeneration score: ≥ 2	15	46 (3-634)
No band at 10 kDa	45	214 (2-1460)
Presence of band at 10 kDa	7	22 (3-365)
No band at 15 kDa	33	244 (2-1460)
Presence of band at 15 kDa	19	122 (2-1190)
UPC < 2.0	6	164 (3-1285)
UPC ≥ 2.0	46	183 (2-1460)
sCr < 1.4	16	192 (2-1460)
sCr ≥ 1.4	36	42 (2-1285)

LM, light microscopy; TI, tubulointerstitial; kDa, kilodalton; UPC, urine protein:creatinine; sCr, serum creatinine.

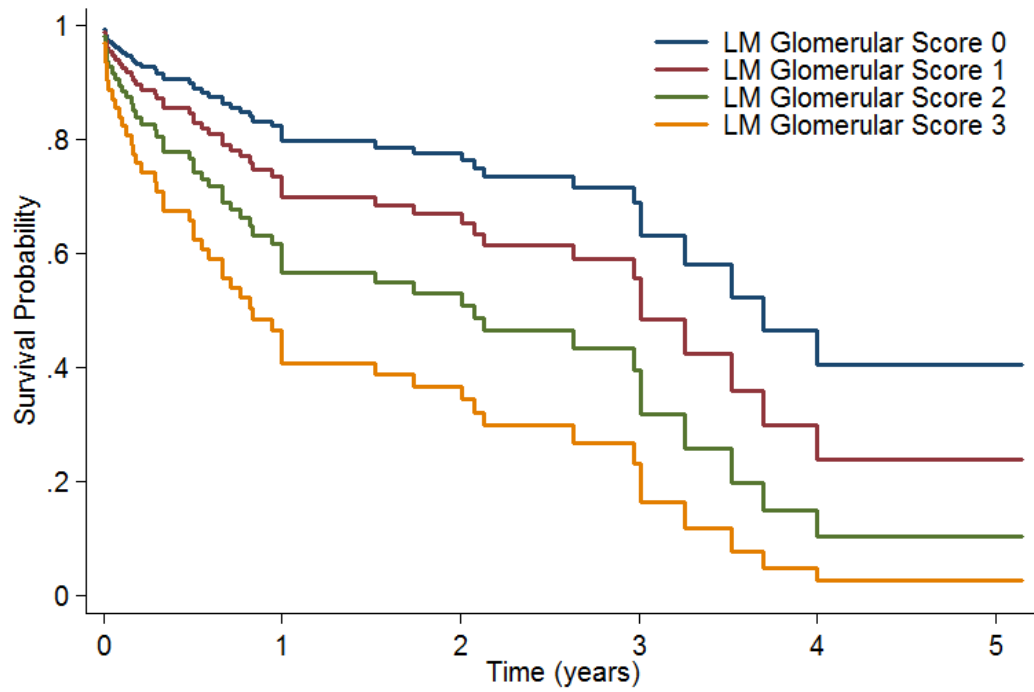


Figure 21. Probability of survival for a dog at median age (7 years) at different light microscopy (LM) glomerular damage scores.

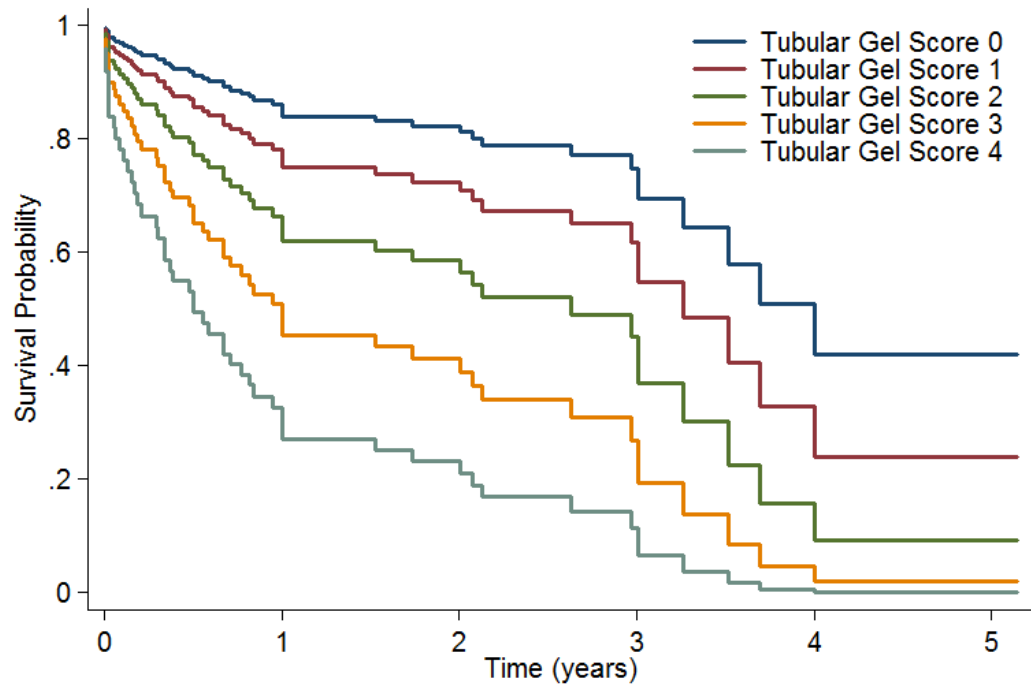


Figure 22. Probability of survival for a dog at median age (7 years) at different tubular gel scores.

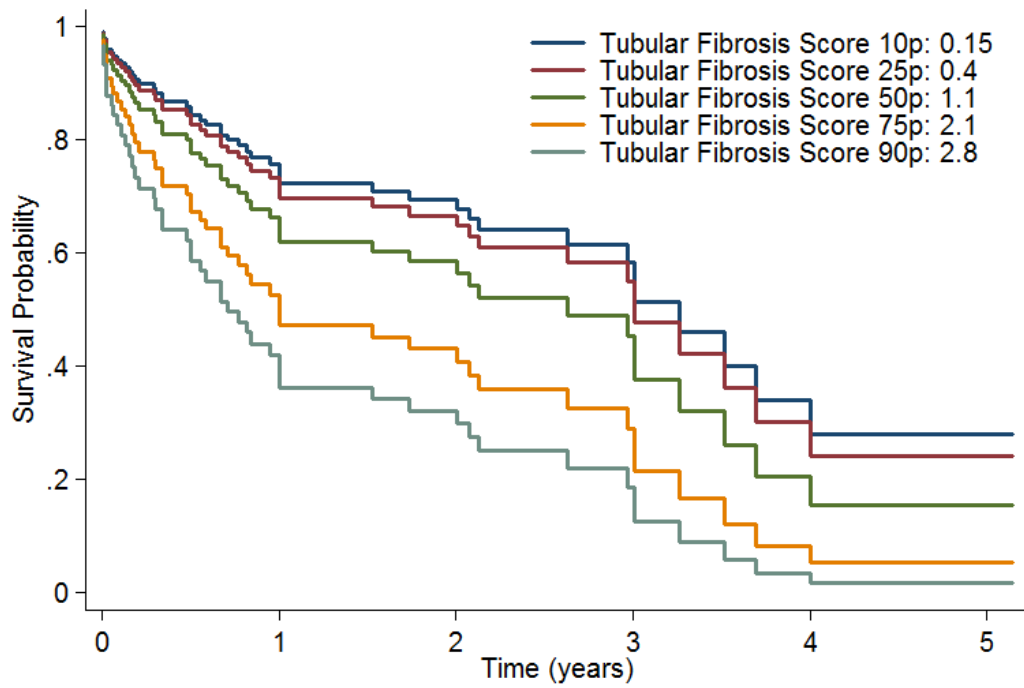


Figure 23. Probability of survival for a dog at median age (7 years) at different tubular fibrosis scores. 10p: 10th percentile; 25p: 25th percentile; 50p: 50th percentile; 75p: 75th percentile; 90p: 90th percentile.

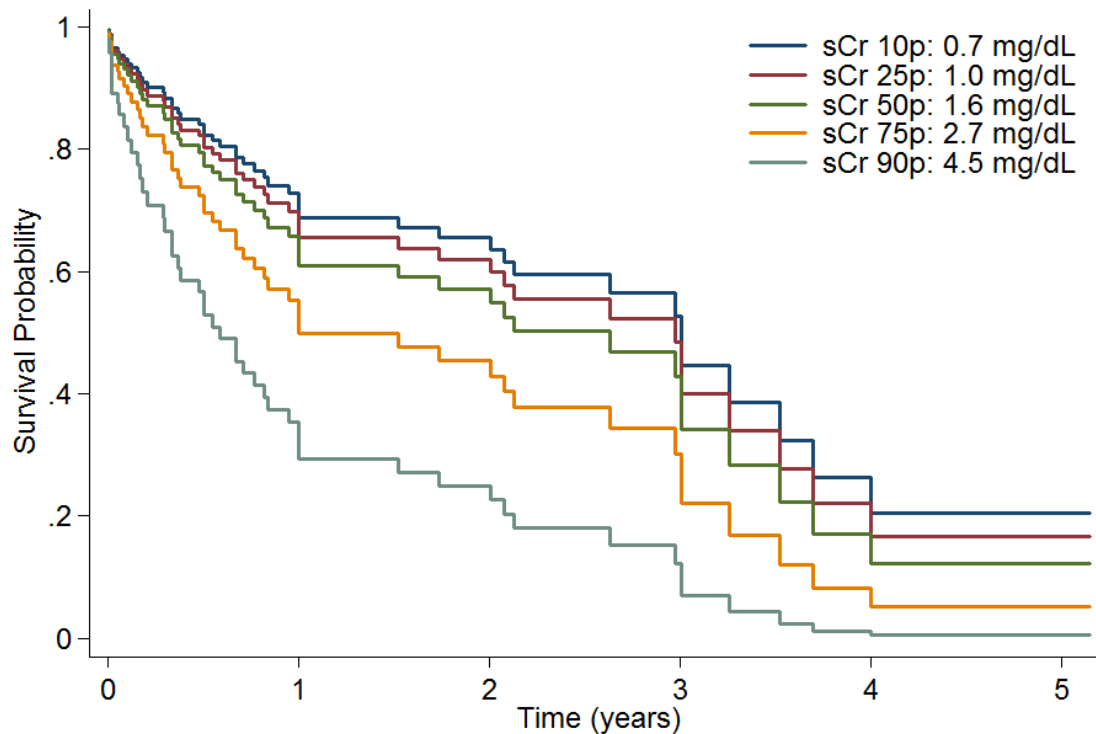


Figure 24. Probability of survival for a dog at median age (7 years) at different starting values of serum creatinine (sCr). 10p: 10th percentile; 25p: 25th percentile; 50p: 50th percentile; 75p: 75th percentile; 90p: 90th percentile.

Discussion

This study evaluated glomerular and tubular urine protein banding patterns and gel scores generated from Bis-Tris gel electrophoresis run on urine samples from 210 dogs with renal disease (predominantly proteinuric CKD) and 11 healthy, non-proteinuric dogs. Glomerular gel scores had excellent sensitivity and specificity for detection of glomerular damage, and tubular gel scores had good sensitivity and excellent specificity for detection of TI damage when the presence of histologic glomerular and TI damage were set as the gold standards in the respective analyses.

There was a strong correlation between all (glomerular and tubular) gel and biopsy scores. Glomerular gel scores were moderately correlated with severity of glomerular damage as assessed by LM and moderately (approaching strongly) correlated with glomerular damage as assessed by TEM. Tubular gel scores were weakly to moderately correlated with the degree of TI damage on renal biopsy, depending on the component of TI damage assessed (fibrosis, atrophy, degeneration, or chronic inflammation), with tubular degeneration having the highest correlation with tubular gel scores. LM glomerular biopsy, tubular gel, TI fibrosis, and tubular degeneration scores, presence of bands at 10 and 15 kDa, sCr, and UPC were all significantly associated with an increased risk of an earlier death due to renal disease. Our findings support that Bis-Tris gel electrophoresis of canine urine can predict presence of glomerular and tubular damage and gel scores might be useful to inform severity of such damage and prognosis in dogs with proteinuric kidney disease.

The few small differences observed in the gel scores on inter-run analysis could be explained by subjectivity of the scoring, particularly given that the scoring incorporated band intensity, and user experience influenced several factors that contributed to the analysis. For that reason, as objective a scoring system as possible was defined and associated with an example gel image to help enhance future scoring reproducibility. Although use of the glomerular and tubular gel scoring algorithm generally provided repeatable results in this study, it could not eliminate disagreement in scoring the degree of glomerular and tubular proteinuria. In the future, methods to develop a more objective scoring system could be considered.

In our experience evaluating urine protein banding patterns, we have appreciated several clinical factors that must be considered when scoring gels, including the presence of renal maldevelopment, neutering status, hematuria, pyuria, and minimally proteinuric samples. Renal maldevelopment usually presents with mild proteinuria, and much of the kidney is no longer producing urine. Consequently, the gel banding patterns do not reflect the severity of the kidney histology in these cases. Intact males often have protein bands at approximately 8-9.5 kDa, 15-16 kDa, and/or 26-27 kDa (Figure 25). These bands are thought to be prostatic fluid proteins, one of which has previously been determined to be arginine esterase.^{20,30,170-172} These prostatic fluid protein bands vary in intensity or sometimes are not present at all in intact males. Because the bands migrate into the LMW region of the gel they can confound assessment of tubular damage if not accounted for. Marked hematuria can result in a slightly hazy or smudged LMW band at approximately 14 kDa and an increased number of IMW and HMW bands, especially above 200 kDa (Figure 25). Similar to hematuria, pyuria increases the number of LMW, IMW, and possibly HMW bands (Figure 25). Therefore, presence of either hematuria or pyuria could alter both glomerular and tubular gel scores. Because of this, samples with evidence of active urine sediments were excluded from analysis in this study. Minimally proteinuric samples tend to have protein bands that are faint on the gels after staining, which makes analysis difficult. To combat this, minimally to mildly proteinuric samples (UPC < 1) are diluted according to the USG but are also run without diluting the urine, which helps to visualize faint bands not observed when diluted based on USG. The staining protocol used can also alter the number and intensity of protein bands seen.

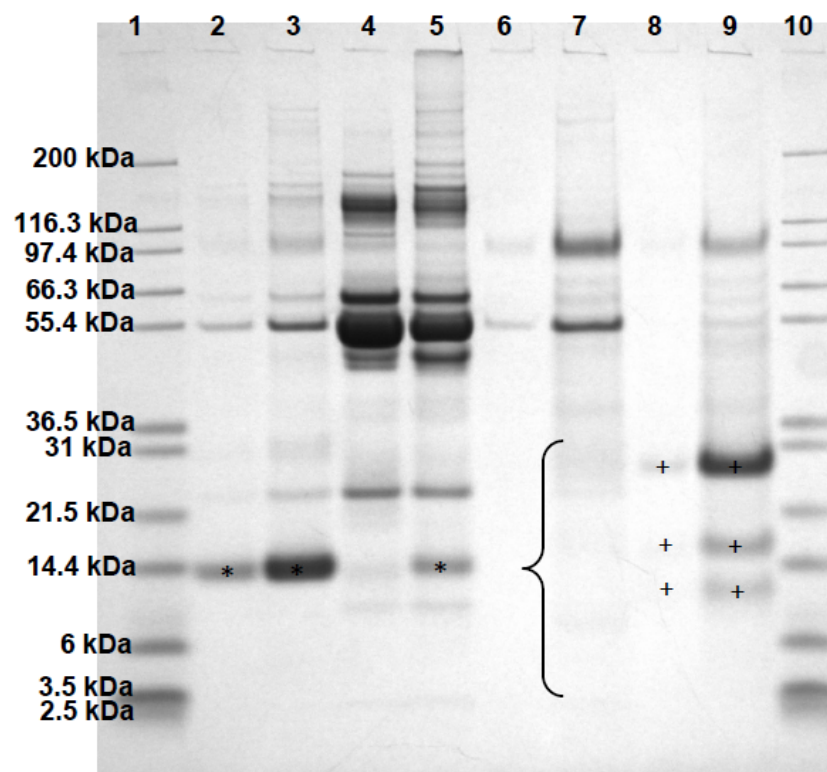


Figure 25. Representative urine samples from dogs with hematuria, pyuria/bacteriuria, and a normal intact male resolved using gel electrophoresis to demonstrate the effect of non-renal disease on urinary protein banding patterns.

Lanes 1 and 10: Molecular weight standards.

Lanes 2 and 3: Urine sample from a healthy, non-proteinuric dog with EDTA blood spiked into the urine sample at a ratio of 500 μ l urine + 1 μ l whole EDTA blood. A prominent hemoglobin band at approximately 14 kDa is the classic feature of hematuria. Additional LMW, IMW, and HMW proteins can be identified that were not present prior to addition of whole blood. The urine sample was diluted based on USG in lane 2 but was not diluted in Lane 3, in which bands are more prominent.

Lanes 4 and 5: Urine samples from a proteinuric dog with glomerular and tubular proteinuria. The sample in lane 5 was collected immediately post-biopsy while the sample collected nearly 2 weeks later (lane 4) had an inactive urine sediment. The 14 kDa hemoglobin band and several additional HMW proteins are evident in the sample in lane 5.

Lanes 6 and 7: Urine sample from an otherwise clinically healthy dog with a urinary tract infection (both pyuria and bacteriuria were present). The sample in lane 6 was diluted based on USG and, although difficult to appreciate in the digital image, demonstrated faint LMW, IMW, and HMW protein bands on the gel. In lane 7, the urine sample was not diluted and more clearly demonstrates the additional LMW, IMW, and HMW protein bands that can be seen with urinary tract infections.

Lanes 8 and 9: Urine sample from a healthy intact male dog. The sample in lane 8 was diluted based on USG and demonstrates faint LMW protein bands at approximately 9 kDa, 15 kDa, and 26 kDa, consistent with prostatic fluid proteins. The sample was not diluted in lane 9, demonstrating more clearly the prominent prostatic fluid proteins bands.

* denotes hemoglobin protein band.

+ denotes prostatic fluid proteins.

{ denotes LMW bands seen with pyuria/bacteriuria.

kDa, kilodalton; HMW, high molecular weight; LMW, low molecular weight; USG, urine specific gravity.

Although silver staining has been used in several studies assessing proteinuria and renal damage in humans, dogs, and rats,^{21,28,163} we opted to use a Coomassie-based stain that is highly sensitive, detecting as little as 3 to 6 ng of protein according to the manufacturer. Silver staining is also highly sensitive,¹⁷³ but unlike Coomassie-based stains, band intensity on silver stain is an unreliable indicator of the amount of protein in the urine. Furthermore, silver staining is technically more difficult to perform than Coomassie-based staining.

The sensitivity and specificity of the glomerular gel scores for glomerular damage were both excellent, and the sensitivity was greater than that for any of the UPC cutoffs considered (≥ 0.5 , 1, or 2) for detecting glomerular damage. $\text{UPC} \geq 2$ is often considered to be a marker of glomerular damage;^{12,124} however, presence of glomerular damage will certainly be missed in some cases if $\text{UPC} \geq 2$ is used as the criteria for glomerular damage and a renal biopsy is not obtained. Therefore, in cases where a renal biopsy is not feasible, performing Bis-Tris gel electrophoresis on a urine sample might provide additional support for presence of glomerular damage, particularly in urine samples with minimal to mild proteinuria. Similarly, the sensitivity and specificity of the tubular damage score for detection of TI damage were good and excellent, respectively, and the sensitivities were higher than those for either a $\text{sCr} \geq 1.4 \text{ mg/dL}$ or $\text{sCr} \geq 1.4 \text{ mg/dL} + \text{USG} < 1.035$, which are commonly used parameters of biomarkers to predict presence of tubular insult. This supports that use of $\text{sCr} \geq 1.4 \text{ mg/dL}$ is insensitive for detecting tubular damage in dogs. Of important note, nearly all clinical cases had at least minimal to mild glomerular and tubular damage; thus, when calculating specificity for

all variables, there were very few true negatives and no false positives which caused specificity to be 100%. It is possible that this high specificity would not be accurate in another population, and therefore, additional studies to verify specificity of the gel scoring system are needed.

Despite the high sensitivities and specificities for glomerular and tubular gel scores for detection of glomerular and TI damage, the gel scores were each only moderately correlated with severity of damage based on the individual glomerular and TI damage scores as determined on biopsy. The glomerular gel score had the highest correlation with the glomerular biopsy score based on TEM, which approached a strong correlation. This is in contrast to the correlation of the glomerular gel score with the glomerular biopsy score based on LM, which was nearing a weak correlation. This suggests that the glomerular protein banding pattern is more indicative of the degree of damage to the glomerular basement membrane ultrastructure. Certainly, it is expected that even minimal changes to the glomerular basement membrane and podocytes could result in altered protein filtration but might not be detectable on LM. One theorized reason that the severity of proteinuria as assessed on gel electrophoresis is not more consistent with the severity of glomerular damage on biopsy is that in cases with marked sclerosis of glomeruli, sclerotic glomeruli might not contribute to protein filtration whereas glomeruli with a lesser degree of damage are still able to contribute to urine production.

The modest correlation of the tubular gel score with the individual or composite TI damage scores on biopsy was expected. Many factors contribute to the number of

bands present in the LMW protein region besides decreased reabsorption of LMW proteins by damaged tubular epithelial cells. It is theorized that glomerular proteinuria, especially when marked, contributes to the number of tubular protein bands due to heavy and light chains of HMW proteins migrating in the LMW region²³⁻²⁵ and due to overload proteinuria resulting in competition of proteins for endocytic receptors and prevention of complete reabsorption of LMW proteins by functional tubular epithelial cells.¹⁵ Indeed, tubular gel scores often overestimated the degree of TI damage, and this discrepancy between gel and biopsy scores can be seen in Table 11 and Figure 19. Conversely, TI damage can demonstrate a patchy distribution throughout the kidney, in which case a biopsy can either under- or over-estimate the overall severity of TI damage. In these cases, the gel score can actually be more representative of overall kidney damage. Additionally, it is possible that only minimal to mild TI damage on LM is needed to cause functional tubular changes as represented by a decreased ability to reabsorb proteins. This would also help explain the discrepancy between the gel and biopsy scores.

The survival analysis results in this study were interesting. While the tubular gel score and sCr were both associated with an increased risk of death due to renal disease, the hazard ratio was greater for the tubular gel score than for sCr. The use of the tubular gel score for assessment of prognosis might be useful in addition to monitoring sCr, especially in cachectic patients or those with limited muscle mass, given the predilection of sCr for overestimation of glomerular filtration rate in such patients.¹⁷⁴ Based on previous studies that have determined the association of specific LMW protein bands

with severe TI damage,^{26,28} we were interested in the association of these particular bands with survival in this cohort of dogs. The presence of a band at 10 or 15 kDa was significantly associated with increased risk of death due to renal disease, and in fact, a band at 10 kDa had the highest HR noted for any biomarker or score evaluated in the survival analysis. Furthermore, there was a difference in median survival time of approximately 6 months between dogs with and without a 10 kDa band. It is important, however, to note the differences in methodologies between this and previous studies and that proteins might run at slightly different molecular weights on different types of gels. Therefore, further investigation is needed into the ability to use specific urinary protein bands on Bis-Tris gel electrophoresis as markers of prognosis in dogs with CKD.

The finding that UPC was significantly, albeit barely, associated with an increased risk of death due to renal disease is in line with findings from a previous study by our group as well as a report of the prognostic ability of UPC and the association of a $UPC \geq 1.0$ at initial evaluation with increased risk of uremic crisis or death in azotemic dogs with CKD.^{12,126} However, when examining median survival times in dogs with UPC above or below 2.0, there was actually a slightly shorter survival time in dogs with the lower UPC values. This is likely due to the low number of cases in the study that had minimal to mild proteinuria and that also had outcome data available, and thus it is possible that these results are not completely accurate and should be reconsidered with a larger population of dogs with more outcome data. Furthermore, dogs with maldevelopment or juvenile onset CKD or dogs with marked TI disease can have minimal proteinuria but faster onset of renal failure and time to death, and the inclusion

of these cases likely contributed to the shorter survival time in dogs with UPCs below 2.0. Finally, in all evaluations performed, age as a covariate was significantly associated with an increased risk of death, and thus, increasing age, not surprisingly, contributes to an increased likelihood of death due to renal disease.

High reproducibility was observed for the method of urine gel electrophoresis described in this study, with both consistent scoring and numbers of protein bands generated for individual samples between runs. The few small differences in the total number or intensity of protein bands on inter-run analysis of an individual sample can be attributed to: 'spill over' from one sample into adjacent lanes because of the high protein content; under- or over-staining of the gel; and operator error (e.g., pipetting and dilution errors). Due to the multitude of factors that can alter gel appearance and subsequent analysis of the gel, any gels that ran abnormally, had an abnormal final appearance, or were not suitable for scoring were repeated to obtain a suitable gel for analysis.

Several limitations were present in this study. The main objective of this study was to explore urine protein banding patterns in dogs with CKD that also received a renal biopsy. Most samples submitted were therefore from dogs that were proteinuric with a suspicion for glomerular, rather than tubular, disease, creating a bias for cases with predominantly glomerular damage. Despite this, a broad range of tubular damage scores was assessed in the study. In future studies, it will be important to determine sensitivity and specificity of Bis-Tris gel electrophoresis for detection of TI damage in a cohort with greater numbers of cases with primary tubular damage.

Although the sample size in this study was large, there are several factors that might impact the measure of correlation. First, there were only a few (3) biopsy scores available for normal canine kidney samples, and these were all analyzed only by LM. Thus, there are very few cases with a LM biopsy score of 0 and no cases with a TEM biopsy score of 0. Because the correlation analyses were based on predominantly non-normal samples, this causes a downward bias and a smaller correlation value. Canonical correlation analysis for correlation of composite biopsy and gel scores was performed only for cases that had all biopsy scores available. However, there were several dogs that had an LM but not a TEM biopsy score. In many cases, this occurred because the diagnosis was confirmed with LM, and TEM was not deemed necessary by the submitting clinician (e.g., amyloidosis). Thus, the exclusion of such cases was not random and might have altered the correlation of the composite scores.

Another limitation of this study is that while most urine samples had a corresponding urinalysis with sediment analysis, several cases lacked this information. Most samples were collected at referral veterinary centers that ideally minimized the likelihood of sediment abnormalities. We were also very strict in our criteria of exclusion of cases with evidence of hematuria and/or pyuria/bacteriuria; however, it is possible that cases with minimal hematuria or pyuria/bacteriuria were unknowingly included. Despite this, the number of additional protein bands that are generated with hematuria or pyuria/bacteriuria are limited when based on USG and particularly compared with the overwhelming renal proteinuria observed in most cases, and therefore

it is unlikely that significant changes in glomerular or tubular gel scores would have occurred even with the inadvertent inclusion of cases with active urinary sediments.

Finally, for purposes of survival analysis, the inclusion of dogs that were euthanized is difficult to avoid in veterinary medicine and might skew survival times. However, the inclusion of dogs that were euthanized replicates the situation seen in the clinical setting and therefore is reasonable.

In conclusion, evaluation of urine samples using Bis-Tris gel electrophoresis cannot replace a well collected and analyzed renal biopsy; however, it can complement renal biopsy evaluation and may provide a method to assess for presence and degree of glomerular and TI damage in renal cases where biopsy is not feasible and there is question as to the renal compartment involved. Additionally, while further exploration into the association with prognosis is needed, protein banding patterns, particularly tubular banding patterns, may inform prognosis.

CHAPTER V

CONCLUSIONS

Evaluation of the degree and type of proteinuria in dogs with CKD can better characterize the location and severity of renal disease and predict prognosis. As discussed in the introduction, many studies have evaluated proteinuria in its many forms (including measurements of UPC and specific proteins in the urine in healthy versus CKD dogs), but few before now have considered a panel of urinary protein biomarkers as indicators of the actual degree of renal damage, cause of CKD, and prognosis in a large group of dogs with naturally occurring CKD.

The primary goal of Chapter II was to determine if selected novel (IgG, IgM, RBP, NGAL, and NAG) and conventional (UPC, sCr, and USG) biomarkers can inform clinicians of the severity of glomerular and TI damage as compared to histology. Additional goals of this chapter were to determine if these biomarkers are associated with specific causes of CKD or prognosis in dogs. Several novel and conventional urinary biomarkers and their fractional excretions were found to correlate with the severity of glomerular and/or TI damage. In particular, the HMW proteins IgG and IgM were better correlated with degree of glomerular damage than UPC, while uNAG/c, a marker of tubular damage in AKI, was not correlated with TI damage but was correlated with glomerular damage in this study. This raises the question of the clinical utility of uNAG/c to indicate tubular damage in cases of canine proteinuric CKD. Furthermore, increased uIgM/c and uNAG/c were significantly associated with ICGN, and sCr,

IgM_FE, and TEM glomerular damage scores were highly associated with an increased risk of death due to renal-related causes. The findings in this first study demonstrate that novel urinary biomarkers can support the presence of glomerular or TI damage, help predict the presence of ICGN, and inform prognosis in dogs with CKD.

Chapter III served as a follow-up study to Chapter II with the goal of further exploring the ability of novel and conventional biomarkers to segregate healthy dogs from those with ICGN and non-ICGN causes of CKD, to segregate dogs with different forms of ICGN, to predict ICGN in dogs, and to inform prognosis in dogs with ICGN. Novel and conventional biomarkers were found to be significantly greater in dogs with ICGN or non-ICGN than in healthy dogs, and UPC and uIgM values separate healthy dogs from dogs with ICGN. Furthermore, UPC and urinary IgM and NAG were all significantly higher in dogs with ICGN compared to dogs with non-ICGN causes of renal disease. uIgM demonstrated the greatest ability to detect ICGN, particularly in azotemic dogs, and high cutoff values for uIgM or uIgM/c had specificities for ICGN above 90%. Thus, in dogs with CKD in which renal biopsy is not feasible, assessment of non-invasive urinary biomarkers might provide enough evidence of ICGN to begin a closely monitored trial of immunosuppressant drugs. Similar to our study in Chapter II, IgM_FE was significantly associated with an increased risk of death due to renal disease, confirming the use of this marker as an indicator of prognosis in a larger population of dogs. The findings in this second study support the hypothesis that urinary biomarkers might be useful to distinguish dogs with ICGN from other causes of proteinuric renal disease.

The goal of Chapter IV was to evaluate electrophoretic urine protein banding patterns from healthy dogs and dogs with proteinuric CKD to determine if banding patterns could identify glomerular and/or TI damage and categorize the severity of damage as compared with renal biopsy results. Additionally, similar to Chapters II and III, we sought to determine if urine protein banding patterns could inform prognosis in dogs. This study generated an objective system for scoring the degree of glomerular and tubular proteinuria on electrophoretic gels and found that the glomerular and tubular gel scores had good to excellent sensitivity and excellent specificity for detection of glomerular and/or TI damage. While correlations between gel and biopsy damage scores were generally moderate when glomerular or TI compartments were considered individually, there was a strong correlation between all (glomerular and tubular) gel scores and all biopsy scores. Finally, tubular gel scores and the presence of a LMW band at 10 or 15 kDa were significantly associated with increased risk of death due to renal disease and shorter survival times were noted for cases with higher tubular gel scores or with a LMW band at 10 or 15 kDa. The findings in this study support that gel electrophoresis of canine urine can detect glomerular and tubular damage, and gel scores and banding patterns can inform prognosis in dogs with proteinuric kidney disease but cannot completely replace a renal biopsy for analysis of severity of renal damage.

One of the overall goals of the studies in this dissertation was to expand on some of the initial exploration work in urinary protein biomarkers performed in a colony of dogs with XLHN that leads to juvenile onset CKD and determine the utility of these minimally invasive markers in dogs that were representative of a true clinical patients.

The results from these 3 studies are promising for the future use of conventional and novel urinary biomarkers and electrophoretic techniques to inform clinicians and owners about the severity and type of renal disease and prognosis in dogs with proteinuric CKD. Several future directions should be considered following these results. Exploration into biomarkers that are specific for glomerular disease should be considered. While several of the biomarkers studied for this dissertation are considered “glomerular” biomarkers, they are certainly not specific, and there is a push in human medicine to identify non-invasive markers that are specific for glomerular injury. It is possible that using global protein, mRNA, or small RNA profiling will uncover markers that are more specific for different compartments of the kidney and for different etiologies of disease.

Furthermore, while these studies identified the ability of biomarkers like urinary IgM to identify ICGN in dogs, prior to use in a clinical setting, measurement of urinary IgM should be applied in a new cohort of dogs with renal diseases to determine if the predictive ability remains the same and if it is influenced by or predicts responsiveness to therapy. A high throughput technique for the measurement of uIgM (e.g., an immunoassay based platform such as the Luminex singleplex or multiplex assays by ThermoFisher Scientific) should also be explored and the sensitivity and specificity of the method should be calculated to determine if uIgM will truly be useful and cost effective in a clinical setting. Additionally, it would be ideal to determine the mechanisms behind elevated urinary IgM in dogs with ICGN. One possible theory is that damage to the glomerular basement membrane is more severe in dogs with immune complex deposition such that very large proteins can be filtered through the glomerular

filtration barrier. Another theory is that portions of the deposited immune complexes, including IgM, are shed into the urine filtrate. Along these lines, it would be beneficial to explore proteins that are specifically related to the pathogenesis of development of ICGN and its different forms and to determine if they would be useful as diagnostic biomarkers. Thus, while these studies have generated answers to the originally posed hypotheses, they have also raised additional questions for future research in CKD that may be applicable to both veterinary and human medicine.

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APPENDIX A*

Supplemental Tables for Chapter I

Table A-1. Urinary Biomarkers of Glomerular Damage/Dysfunction in Small Animals¹⁷⁴

<u>Renal Biomarker</u>	<u>Location of Production</u>	<u>Type of Protein/Biomarker</u>	<u>Validation / species</u>	<u>Values in Healthy Animals</u>	<u>Affected in AKI, CKD, or Both</u>	<u>Non-Renal Influences</u>
Albumin	Hepatocytes	Negative acute phase protein	Dogs ⁵⁰	<i>Dogs: uAlb/c: < 296.5 mg/g;^{20,41-43,51,64} Alb FE: 0%⁵¹ Cats: uAlb: 11.2 +/- 8.4 mg/dL¹⁷⁵</i>	AKI: Dogs ^{40-42,52,53} CKD: Dogs, ^{43-46,50,51} cats ^{175,176}	Treatment with hydrocortisone (dogs) ³⁸
C-Reactive Protein	Hepatocytes	Positive acute phase protein	Dogs ^{35,177}	<i>Dogs: uCRP/c: 1.06 µg/g;¹⁷⁷ below detection limit of immunoassay^{35,39-42,50}</i>	AKI: Dogs ^{35,39-42,177} CKD: Dogs ^{35,177}	
IgA	Plasma cells in spleen, lymph nodes, bone marrow	Antibody; HMW protein		Not detectable in healthy dog urine (Western blot) ²³⁻²⁵	AKI: Dogs ²³⁻²⁵	
IgG	Plasma cells in spleen, lymph nodes, bone marrow	Antibody; HMW protein	Dogs ^{35,37,43}	<i>Dogs: uIgG/c: < 10 mg/g^{35,37,39-42}; in Dogue de Bordeaux - uIgG/c median (range): 1.41 (0.26-23.60) mg/g²⁰</i>	AKI: Dogs ^{23-25,35,39-42} CKD: Dogs ^{36,37,43-46}	Hematuria+; Pyuria/bacteriuria+; Treatment with hydrocortisone ³⁸
IgM	Plasma cells in spleen, lymph nodes, bone marrow	Antibody; HMW protein	Dogs ³⁶		CKD: Dogs ³⁶	Hematuria+; pyuria/bacteriuria+
TXB2	Glomerular mesangial cells and podocytes	Cyclooxygenase lipid metabolite; marker of altered intra-renal hemodynamics	Dogs ³⁵	<i>Dogs: uTXB2/c: < 4.7 µg/g^{35,41,42}</i>	AKI: dogs ^{41,42}	
Transferrin	Primarily liver; other tissues as well	Iron transport protein		<i>Cats: uTf: 0.09 +/- 0.42 mg/dL¹⁷⁵</i>	CKD: Dogs, ^{44,45} Cats ^{175,176}	

+Personal observations

AKI, acute kidney injury; Alb_FE, fractional excretion of albumin; CKD, chronic kidney disease; HMW, high molecular weight; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; TXB2, thromboxane B2; uALB, urinary albumin concentration; uALB/c, urinary albumin/urinary creatinine; uCRP/c, urinary C-reactive protein/urinary creatinine; uIgG/c, urinary immunoglobulin G/urinary creatinine; uTf, urinary transferrin concentration; uTXB2/c, urinary thromboxane B2/urinary creatinine.

* Tables A-1, A-2, and A-3 are reprinted with permission from Renal Biomarkers in Domestic Species by J. Hokamp and M. Nabity, 2016. Veterinary Clinical Pathology 45, 28-56, Copyright 2016 by American Society for Veterinary Clinical Pathology.

Table A-2. Urinary Protein and Enzyme Biomarkers of Tubular Damage/Dysfunction in Small and Large Animals¹⁷⁴

<u>Renal Biomarker</u>	<u>Location of Production</u>	<u>Type of Protein/Biomarker</u>	<u>Mechanism for altered excretion</u>	<u>Validation / species</u>	<u>Values in Healthy Animals</u>	<u>Affected in AKI, CKD, or Both</u>	<u>Non-Renal Influences</u>
<u>Urinary Proteins</u>							
Clusterin	Renal tubules	Glycoprotein	Increased production	Dogs ¹⁷⁸	<i>Dogs</i> : <u>uClu/c</u> : 0.27 (unitless); ¹⁷⁸ <u>uClu</u> : 38.5ng/ml; ¹⁷⁸	AKI: Dogs ^{52,178}	
Cystatin C	All nucleated cells	LMW protein and proteinase inhibitor	Decreased reabsorption	Dogs ¹⁷⁹ Cats ¹⁸⁰	<i>Cats</i> : below limit of quantification of assay ¹⁸¹	AKI: Dogs ⁵³ CKD: Dogs, ^{179,182} cats ^{180,181}	Diabetes in cats ¹⁸¹
KIM-1	Renal tubules	Glycoprotein	Increased production		Undetectable in healthy cats ¹⁸³	Both, but mostly with acute processes AKI: Dogs, ⁵² cats, ¹⁸³ a lamb ¹⁸⁴	
NGAL	Neutrophils, kidney, bronchus, stomach, small intestine, pancreas, prostate gland, thymus	LMW glycoprotein	Decreased reabsorption and increased production	Dogs ^{36,37,81,82}	<i>Dogs</i> : <u>uNGAL/c</u> : < 6 µg/g; ^{37,81,84} <u>uNGAL</u> : < 21.2 ng/mL ^{78,81-83,86}	AKI: Dogs ^{52,53,78,82,84-86,88,90,91,185} CKD: Dogs ^{36,37,78,82-84,86}	Inflammation/urinary tract infection/pyuria (dogs); ^{78,81,84} <u>uNGAL/c</u> decreased with body mass (dogs); ⁸¹ Age (dogs) ^{37,81}
RBP	Primarily liver; also other organs (kidney, lungs, spleen, brain, stomach, heart, skeletal muscle)	Vitamin A carrier protein	Decreased reabsorption	Dogs ^{35,37,63} Cats ⁶⁶	<i>Dogs</i> : <u>uRBP/c</u> : < 0.15 mg/g; ^{20,35,37-43,46,50,51,65} <i>Cats</i> : Undetectable ⁶⁶ <i>Sheep</i> : Undetectable ¹⁸⁶	AKI: Dogs, ^{35,40,42,69} sheep; ¹⁸⁶ CKD: Dogs, ^{35-37,43-46,51,69,70} cats ^{66-68,176}	Marked hematuria; ⁶⁴ Pyuria/bacteriuria (RBP_FE)*; Negative correlation with age in young adolescent dogs; ³⁷ Treatment with hydrocortisone (dogs) ³⁸
THP	Epithelial cells of thick ascending limb of loop of Henle and distal convoluted tubule	Glycoprotein	Decreased production	Cats ¹⁸⁷	<i>Dogs</i> : <u>uTHP/c</u> : 10 – 65 mg/g ¹¹³ <i>Cats</i> : <u>uTHP</u> : 49.2 +/- 35.5 µg/ml ¹⁸⁷	CKD: Dogs, ^{44,63,69,112,113} cats ¹⁷⁶	Urolithiasis (cats) ¹⁸⁷
<u>Urinary Enzymes</u>							
AAP	Renal tubular brush border enzyme	Enzyme	Released from brush border	Dogs ¹⁰¹	<i>Dogs</i> : <u>uAAP/c</u> : 0.7-9.0 U/g ¹⁰¹	AKI: Dogs ⁵²	
ALP	Renal tubular brush border enzyme, highest in renal proximal convoluted tubule; Also in tubular lysosomes	Enzyme	Released from brush border		<i>Dogs</i> : <u>uALP/c</u> : 0.25 +/-1.17 U/g; ¹⁸⁸ <u>uALP</u> : 5.8 (0.4-12) U/L/mmol ¹⁸⁹ <i>Horses</i> : <u>uALP/c</u> : 6.7 +/- 3.9 IU/g; ¹⁹⁰ <u>uALP</u> : 10.2 +/- 4.0 IU/L; ¹⁹⁰ < 3.55 U/mmol ^{191,192}	AKI: Dogs, ^{65,189,193} sheep ¹⁹⁴	
Cauxin	Proximal straight tubular cells in cats	Carboxylesterase protein	Uncertain whether increased or decreased with tubular damage	Cats ¹⁹⁵		CKD: Cats ^{176,195,196}	
GGT	Renal tubular brush border enzyme, highest in renal proximal straight tubule	Enzyme	Released from brush border	Horses ¹⁹⁷	<i>Dogs</i> : <u>uGGT/c</u> : < 42 U/g; ^{103,108,188,198-200} 3.4 (0.6-6.0) U/L/mmol ¹⁸⁹ <u>uGGT</u> : 6-112 U/L ²⁰⁰ <i>Cats</i> : <u>uGGT/c</u> : < 36.4 U/g ²⁰¹ <i>Horses</i> : <u>uGGT/c</u> : < 36.54 U/g; ^{190,191,197,202} 0.33 +/- 0.8 U/L/mmol ²⁰³ <u>uGGT</u> : 3.3 +/- 3.0 IU/L; ¹⁹⁰ < 1.85 IU/mmol ^{191,192,197} <i>Calves</i> : <u>uGGT</u> : 3.2 +/- 2.6 U/µmol ²⁰⁴	AKI: Dogs, ^{65,106,189,193,200,205} horses, ^{206,207} sheep ¹⁹⁴	
NAG	Lysosomal enzyme in proximal renal tubules and other tissues; 2 renal forms: NAG-A and NAG-B; circulates in the serum	Enzyme	Cellular leakage (+/- glomerular damage, possibly due to lysosomal turnover and/or leakage through glomerular filtration barrier)	Dogs ^{37,50,64,101} and cats ²⁰⁸	<i>Dogs and cats</i> : <u>uNAG/c</u> : < 11 U/g ^{14,101,103,201} ; Dogue de Bordeaux – <u>uNAG/c</u> median (range): 5.74 (2.75 – 13.396) U/g ²⁰	AKI: Dogs, ^{41,42,52,53,88,90,102,105,106} cats, ²⁰⁹ sheep ¹⁹⁴ CKD: Dogs, ^{36,37,43,50} cats ²⁰⁹	Alkaline urine (reduced activity); ²⁰¹ Hematuria and hemoglobinuria (inaccurate measurement due to similar color of hemoglobin to m-crescol purple released by some substrates); ²⁰¹ Gender (higher in male dogs) ^{101,103}

*Personal observations

AAP, alanine aminopeptidase; AKI, acute kidney injury; ALP, alkaline phosphatase; CKD, chronic kidney disease; GGT, gamma glutamyl-transpeptidase; KIM-1, kidney injury molecule-1; LMW, low molecular weight; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; RBP, retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; THP, Tamm-Horsfall protein; uAAP/c, urinary alanine aminopeptidase/urinary creatinine; uALP, urinary alkaline phosphatase concentration; uALP/c, urinary alkaline phosphatase/urinary creatinine; uClu/c, urinary clusterin/urinary creatinine; uGGT, urinary gamma glutamyl-transpeptidase; uGGT/c, urinary gamma glutamyl-transpeptidase/urinary creatinine; uNAG/c, urinary N-acetyl-β-D-glucosaminidase/urinary creatinine; uNGAL, urinary neutrophil gelatinase-associated lipocalin concentration; uNGAL/c, urinary neutrophil gelatinase-associated lipocalin/urinary creatinine; uRBP/c, urinary retinol binding protein/urinary creatinine; uTHP, urinary Tamm-Horsfall protein concentration; uTHP/c, urinary Tamm-Horsfall protein/urinary creatinine.

Table A-3. Summary of Serum and/or Plasma Renal Biomarkers in Dogs and Cats¹⁷⁴

<u>Renal Biomarker</u>	<u>Location of Production</u>	<u>Type of Protein/Molecule</u>	<u>Type of Biomarker</u>	<u>Validation / Species</u>	<u>Values in Healthy Animals</u>	<u>Affected in AKI, CKD, or Both</u>	<u>Non-Renal Influences</u>
Creatinine	Muscle	Cyclic derivative of creatine	Endogenous indirect GFR marker	Dogs Cats	Dogs: 0.5-1.5 mg/dL ²¹⁰ Cats: 0.8-1.8 mg/dL ²¹⁰ (but depends on breed/species/instrument)	Both	Muscle mass; meat diet; hydration status
Cystatin C	All nucleated cells	LMW protein and proteinase inhibitor	Endogenous indirect GFR marker	Dogs ^{118,120,211,212} Cats ^{213,214}	Dogs: < 2.28 mg/L ^{118-120,182,211,215-217} Cats: < 1.95 mg/L ^{213,214,218,219}	CKD: Dogs, ^{118-120,182,211,215-217} cats ^{213,218,219} (Presumably also AKI)	Obesity and weight loss in dogs ²¹²
SDMA	All nucleated cells	Methylated amino acid (arginine)	Endogenous indirect GFR marker	Dogs ^{122,220,221} Cats ^{123,220,221}	Dogs and cats: < 14 µg/dL ^{122,123,222-226}	CKD: Dogs, ^{122,226} cats ^{123,227,228} (Presumably also AKI)	
FGF-23	Osteocytes and osteoblasts	Phosphaturic hormone	Marker of altered phosphorus metabolism	Cats ²²⁹	Cats: 56-700 pg/mL ²²⁹	CKD: Cats ²²⁹⁻²³²	Dietary phosphorus; ²³¹ hyperthyroidism ²³²
NGAL	Neutrophils, kidney, bronchus, stomach, small intestine, pancreas, prostate gland, thymus	LMW glycoprotein	Endogenous indirect GFR marker	Dogs ⁸²	Dogs: < 21.2 ng/mL ^{82,83,86}	AKI: Dogs ^{82,86} CKD: Dogs ^{82,83,86}	Inflammation ²³³
C-Reactive Protein	Hepatocytes	Positive acute phase protein	Inflammatory marker	Dogs ²³⁴	Dogs: 3.21 mg/l (range: 2.09-8.60 mg/l) ²³⁴	Renal diseases in dogs (AKI versus CKD not specified) ²³⁴	
Homocysteine	All nucleated cells	Amino acid; Intermediate product of methionine metabolism	Endogenous indirect GFR marker	Dogs ^{212,235,236}	Dogs: 4.35 +/- 2.69 µmol/L ²³⁶	AKI: Dogs ²³⁵ CKD: Dogs ^{235,236}	Obesity and weight loss in dogs; ²¹² icterus, severe hemolysis and lipemia; ²³⁵ cardiac disease ²³⁵
Big-Endothelin-1	Blood vessels, lung, other tissues including kidney medulla	Precursor to endothelin-1, a vasoconstrictor peptide	Inflammation	Dogs ²³⁶	Dogs: 6.51 +/- 1.86 pg/mL ²³⁶	CKD: Dogs ²³⁶	Hypertension and systemic inflammation ²³⁶

AKI, acute kidney injury; CKD, chronic kidney disease; FGF-23, fibroblast growth factor-23; GFR, glomerular filtration rate; LMW, low molecular weight; NGAL, neutrophil gelatinase-associated lipocalin; SDMA, symmetric dimethylarginine.

Table A-4. Median (range) for biomarkers comparing pyuric and bacteriuric samples, hematuric samples, and all other samples (i.e., those without pyuria, bacteriuria, or hematuria) from dogs with naturally occurring chronic kidney diseases.

	Pyuria/Bacteriuria		Hematuria		All Other Samples	
	n	Mean (Range)	n	Mean (Range)	n	Mean (Range)
sCr (mg/dL)	8	2.9 (0.9 - 9.5)	14	2.0 (0.4 - 4.6)	179	1.5 (0.3 - 21.8)
USG	8	1.017 (1.014 - 1.026)	15	1.022 (1.004 - 1.042)	180	1.017 (1.003 - 1.048)
UPC Ratio	8	7.0 (0.7 - 23.6)	15	8.0 (2.9 - 26.7)	180	5.0 (0.0 - 36.8)
IgG						
uIgG/c (µg/mg)	8	963 (118 - 9,532)	15	1,286 (261 - 15,223)*	180	554 (1 - 37,649)
sIgG (µg/mL)	7	10,338 (2,235 - 27,077)	10	10,015 (2,637 - 47,627)	137	11,320 (3,199 - 41,798)
IgG_FE %	7	0.3 (0.0 - 4.6)*	10	0.2 (0.0 - 1.7)	137	0.1 (0.0 - 8.6)
IgM						
uIgM/c (µg/mg)	8	23.2 (2.4 - 240.8)*	15	22.4 (5.0 - 546.1)*	180	6.4 (0.3 - 557.0)
sIgM (µg/mL)	7	2,144 (421 - 10,879)	10	1,776 (645 - 13,978)	137	2,864 (867 - 20,070)
IgM_FE %	7	0.042 (0.007 - 0.122)*	10	0.020 (0.002 - 0.163)*	137	0.003 (0.000 - 0.172)
RBP						
uRBP/c (µg/mg)	8	59.3 (0.7 - 294.6)	15	12.0 (1.1 - 111.0)	180	8.7 (0.0 - 1013.4)
sRBP (µg/mL)	7	83.9 (33.2 - 111.8)	10	76.4 (29.9 - 160.6)*	137	106 (23 - 414)
RBP_FE %	7	6.6 (0.1 - 11.2)*	10	0.4 (0.0 - 7.7)	137	0.1 (0.0 - 24.9)
NGAL						
uNGAL/c (ng/mg)	7	335 (74 - 1,957)*	14	98.7 (43.3 - 2,535.0)	153	93.6 (0.0 - 1,533.4)
sNGAL (ng/mL)	6	36.2 (22.4 - 84.7)*	9	12.4 (3.8 - 190.0)	124	12.1 (2.4 - 149.1)
NGAL_FE %	6	43.0 (1.0 - 227.2)	9	16.7 (1.0 - 145.9)	124	10.8 (0.0 - 505.9)
uNAG/c (U/g)	8	33.6 (2.6 - 92.5)	14	16.0 (3.8 - 98.7)	170	13.5 (0.4 - 427.7)

Pyuria/bacteriuria: >10 WBC/40× field, presence of bacteriuria, or both; Hematuria: >100 RBC/40× field; *P < 0.05 significant difference between samples with pyuria/bacteriuria or hematuria and all other samples; sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; sIgG, serum immunoglobulin G; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; sIgM, serum immunoglobulin M; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

APPENDIX B*

Supplemental Materials and Methods for Chapter II

*Assay Validation*¹²

For the immunoglobulin M (IgM) assay, intra- and interassay variability, dilutional linearity, and spiking recovery were determined using canine urine samples. For the neutrophil gelatinase-associated lipocalin (NGAL) assay, interassay variability and dilutional linearity were determined using canine urine samples. Intraassay variability was determined by analyzing 10 repetitions of 3 samples with low, middle, and high concentrations within a single run. Interassay variability was determined by analyzing 3 samples with low, middle, and high analyte concentrations in 8 (IgM) or 13 (NGAL) consecutive runs. For dilutional linearity, 3 – 4 samples with low, middle, and high analyte concentrations were serially diluted. For spiking recovery, a known amount of protein standard was added to 6 samples with known IgM concentrations. Additionally, 5 canine urine samples with low, middle, and high IgM concentrations were mixed in various combinations. Intra- and interassay variability were calculated using coefficient of variation (CV). Dilutional linearity and spiking recovery were calculated using observed to expected ratios (O/E%).

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Supplemental Tables for Chapter II

Table A- 5. Glomerular scoring system based on light microscopy.¹²

Score	Lesions
0	Normal glomeruli or mild mesangial expansion
1	Mild or focal lesions: focal segmental glomerulosclerosis in small numbers of glomeruli; obsolescence of a small number of glomeruli; mild or early ICGN; mild or early amyloidosis (most capillary loops remain patent); other non-specific glomerular lesions (e.g., mesangial expansion; mild mesangial cell proliferation; glomerular atrophy; podocyte hypertrophy without sclerosis; fetal glomeruli; glomerular hypertrophy)
2	Moderate lesions: focal segmental sclerosis in many glomeruli; moderate ICGN with GBM remodeling and secondary sclerosis; moderate amyloidosis (effacement of about ½ of the capillary loops by amyloid); other non-specific glomerular lesions (e.g., glomerular obsolescence/atrophy with associated secondary compensatory hypertrophy and sclerosis)
3	Severe lesions: advanced focal segmental glomerulosclerosis; active or fulminant ICGN with abundant inflammatory cells, crescents or pyknotic nuclear debris; severe amyloidosis (most capillary loops effaced by amyloid); advanced global glomerulosclerosis; end-stage lesions with secondary compensatory hypertrophy and sclerosis

ICGN, immune complex-mediated glomerulonephritis; GBM, glomerular basement membrane.

Table A-6. Glomerular scoring system based on TEM.¹²

Score	Lesions
0	Normal ultrastructure or minimal, nonspecific/reversible lesions identified in a few TEM images (e.g., segmental foot process effacement, microvillus transformation)
1	Mild, reversible ultrastructural lesions (identified in many TEM images but still segmental in distribution): foot process effacement, global podocyte swelling, or global microvillus transformation
2	Moderate irreversible ultrastructural lesions (identified in many TEM images): segmental glomerular basement membrane remodeling, podocyte dropout, scattered/focal immune deposits, mild amyloidosis or global foot process effacement
3	Severe irreversible ultrastructural lesions (identified in most TEM images): global glomerular basement membrane remodeling, marked podocyte dropout, global immune complex deposition, or moderate to severe amyloidosis

TEM, transmission electron microscopy.

Table A-7. Tubulointerstitial scoring system based on light microscopy.¹²

Score	Interstitial Fibrosis*	Tubular Atrophy**	Tubular Degeneration/Necrosis/Regeneration**	Interstitial Chronic Inflammation ⁺
0	None	None	None	None
1	Mild non-diffuse fibrosis: surrounds ≤ 2 tubules	Mild non-diffuse atrophy: ≤ 2 tubules	Mild non-diffuse cell stress: ≤ 2 tubules degenerating and/or regenerating	Mild non-diffuse chronic inflammation: ≤ 2 mononuclear cell foci (<10 cells)
2	Mild diffuse fibrosis: surrounds ≥ 3 tubules	Moderate non-diffuse atrophy: 3 - 5 tubules	Mild non-diffuse cell death: ≤ 2 tubules with necrotic/apoptotic cells	Mild diffuse chronic inflammation: ≥ 3 mononuclear cell foci (<10 cells)
3	Moderate non-diffuse fibrosis: surrounds ≤ 2 tubules	Severe atrophy: all tubules in a field or > 6 tubules	Moderate diffuse cell stress: ≥ 3 tubules degenerating and/or regenerating	Moderate non-diffuse chronic inflammation: ≤ 2 mononuclear cell foci (>10 cells)
4	Moderate diffuse fibrosis: surrounds ≥ 3 tubules	N/A	Moderate diffuse cell death: ≥ 3 tubules with necrotic/apoptotic cells	Moderate diffuse chronic inflammation: ≥ 3 mononuclear cell foci (>10 cells)
5	Severe fibrosis (fibrosis replacing tubules)	N/A	Severe tubular necrosis: entire tubule in field is necrotic or contains cellular cast	Severe chronic inflammation: any field where inflammatory foci replace tubules

For each category, 10 random 40 \times fields for core biopsies and 30 random 40 \times fields for wedges were evaluated.

*Evaluated with trichrome; **evaluated with periodic acid-Schiff; +evaluated with hematoxylin and eosin; N/A, not applicable.

Table A-8. Results from IgM and NGAL assay validation including mean inter- and intra-assay variability for low, middle, and high concentration urine samples, dilutional linearity, and spiking recovery.¹²

	Mean Interassay Variability (CV)			Mean Intraassay Variability (CV)			Dilutional Linearity (O/E%)	Spiking Recovery (O/E%)
	[Low]	[Middle]	[High]	[Low]	[Middle]	[High]		
IgM Assay	10.8%	12.6%	14.6%	2.7%	3.3%	3.4%	95-125%*	87-119%
NGAL Assay	13.4%	13.6%	11.7%	ND	ND	ND	86-118%	N/A

*Dilutional linearity of the IgM assay was acceptable when the values obtained fell within 125-1,000 ng/mL on the standard curve. When values were below this, the final concentration was overestimated by 40 – 130%. IgM, immunoglobulin M; NGAL, neutrophil gelatinase-associated lipocalin; CV, coefficient of variation; O/E%, observed to expected ratio; ND, not performed.

Table A-9. Median (range) for biomarkers within each IRIS stage.¹²

	IRIS Stage 1		IRIS Stage 2		IRIS Stage 3		IRIS Stage 4	
	n	Median (Range)	n	Median (Range)	n	Median (Range)	n	Median (Range)
sCr (mg/dL)	73	0.9 (0.3 - 1.3) ^{abc}	50	1.6 (1.4 - 2.0) ^{de}	41	2.8 (2.1 - 4.9) ^f	15	8.5 (5.3 - 21.8)
USG	73	1.020 (1.003 - 1.048) ^{bc}	50	1.018 (1.003 - 1.045) ^{de}	41	1.013 (1.005 - 1.029)	15	1.013 (1.006 - 1.020)
UPC Ratio	73	5.2 (0.1 - 31.0)	50	6.8 (0.0 - 31.6)	41	3.4 (0.0 - 36.8)	15	3.7 (0.2 - 16.2)
IgG								
uIgG/c (µg/mg)	73	558 (1 – 7,183)	50	625 (1 – 37,649)	41	356 (2 – 11,845)	15	278 (22 – 2,954)
sIgG (µg/mL)	58	11,288 (3,742 – 29,686)	38	11,369 (3,294 – 41,798)	30	9,400(3,576 – 25,399)	11	14,916 (3,199 – 31,108)
IgG_FE %	58	0.043 (0.000 - 0.628) ^{bc}	38	0.086 (0.000 - 3.233)	30	0.114 (0.000 - 5.056)	11	0.157 (0.039 - 8.587)
IgM								
uIgM/c (µg/mg)	73	6.2 (0.8 - 58.2)	50	7.0 (0.3 - 150.6)	41	5.5 (0.5 - 557.0)	15	7.9 (0.4 - 29.7)
sIgM (µg/mL)	58	2,724 (868 – 12,042)	38	2,937 (1,051 – 16,031)	30	3,030 (1,066 – 20,070)	11	2,759 (1,688 – 10,922)
IgM_FE %	58	0.002 (0.000 - 0.021) ^{abc}	38	0.003 (0.000 - 0.027) ^{de}	30	0.008 (0.001 - 0.089)	11	0.015 (0.002 - 0.172)
RBP								
uRBP/c (µg/mg)	73	1.9 (0.0 – 1,013.4) ^{bc}	50	8.0 (0.0 - 699.0) ^e	41	17.4 (0.1 - 427.5) ^f	15	38.4 (9.5 - 306.1)
sRBP (µg/mL)	58	94 (27 - 258) ^c	38	101 (23 - 281) ^e	30	113 (24 - 238)	11	153 (44 - 414)
RBP_FE %	58	0.0 (0.0 - 0.8) ^{abc}	38	0.1 (0.0 - 3.2) ^{de}	30	0.4 (0.0 - 18.0) ^f	11	5.1 (0.5 - 24.9)
NGAL								
uNGAL/c (ng/mg)	62	74 (0 – 1,533)	40	93 (4 - 493)	39	103 (1 - 912)	11	140 (11 - 677)
sNGAL (ng/mL)	53	9.9 (2.5 - 65.7) ^{bc}	33	13.5 (2.4 - 73.2)	30	14.5 (2.6 - 149.1)	8	30.5 (3.2 - 62.9)
NGAL_FE %	53	5.8 (0.0 - 115.2) ^{abc}	33	11.1 (1.0 - 43.3) ^{de}	30	17.9 (1.6 - 82.8) ^f	8	49.5 (10.8 - 505.9)
uNAG/c (U/g)	70	14.4 (2.0 - 253.1)	46	12.4 (1.3 - 109.9)	40	10.7 (0.4 - 427.7)	13	12.8 (0.4 - 37.7)

Significant differences ($P < 0.05$) exist for biomarkers between IRIS stages ^a1 and 2, ^b1 and 3, ^c1 and 4, ^d2 and 3, ^e2 and 4, ^f3 and 4 as determined by linear regression modeling comparing groups. IRIS, International Renal Interest Society; sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; sIgG, serum immunoglobulin G; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; sIgM, serum immunoglobulin M; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

Table A-10. Correlation among biomarkers for dogs with naturally occurring CKD.¹²

	USG	UPC	uIgG/c	sIgG	IgG_FE	uIgM/c	sIgM	IgM_FE	uRBP/c	sRBP	RBP_FE	uNGAL/c	sNGAL	NGAL_FE	uNAG/c
sCr	-0.32**	-0.15*	-0.12	0.02	0.31**	-0.01	0.09	0.59**	0.41**	0.19*	0.62**	0.09	0.26**	0.46**	-0.11
USG		0.24**	0.21**	-0.06	0.08	0.17*	0.06	-0.06	-0.11	-0.18*	-0.13	-0.06	-0.09	-0.16	0.24**
UPC			0.90**	-0.25**	0.65**	0.65**	0.06	0.42**	0.46**	0.11	0.46**	0.44**	0.13	0.00	0.73**
uIgG/c				0.00	0.90**	0.72**	0.18*	0.55**	0.42**	-0.04	0.42**	0.59**	0.19*	0.49**	0.68**
sIgG					-0.24**	0.06	0.49**	-0.13*	-0.22**	-0.06	0.07	-0.04	0.10	-0.09	-0.26**
IgG_FE						0.66**	0.07	0.75**	0.64**	0.05	0.66**	0.62**	0.24**	0.64**	0.66**
uIgM/c							0.52**	0.77**	0.39**	-0.08	0.46**	0.23**	0.23**	0.09	0.29**
sIgM								0.09	0.00	-0.21	0.07	0.12	0.23**	0.03	0.03
IgM_FE									0.66**	0.12	0.74**	0.30**	0.27**	0.32**	0.45**
uRBP/c										0.35**	0.95**	0.62**	0.30**	0.66**	0.44**
sRBP												0.23*	0.27**	0.14	-0.20*
RBP_FE												0.57**	0.30**	0.69**	0.45**
uNGAL/c													0.45**	0.78**	0.50**
sNGAL														-0.02	0.15
NGAL_FE															0.38**

* P < 0.05; ** P < 0.001; Shading depicts correlations with P < 0.001. sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio; uIgG/c, urine immunoglobulin G/urine creatinine; sIgG, serum immunoglobulin G; IgG_FE, fractional excretion of immunoglobulin G; uIgM/c, urine immunoglobulin M/urine creatinine; sIgM, serum immunoglobulin M; IgM_FE, fractional excretion of immunoglobulin M; uRBP/c, urine retinol binding protein/urine creatinine; sRBP, serum retinol binding protein; RBP_FE, fractional excretion of retinol binding protein; uNGAL/c, urine neutrophil gelatinase-associated lipocalin/urine creatinine; sNGAL, serum neutrophil gelatinase-associated lipocalin; NGAL_FE, fractional excretion of neutrophil gelatinase-associated lipocalin; uNAG/c, urine N-acetyl-β-D-glucosaminidase/urine creatinine.

Supplemental Figures for Chapter II

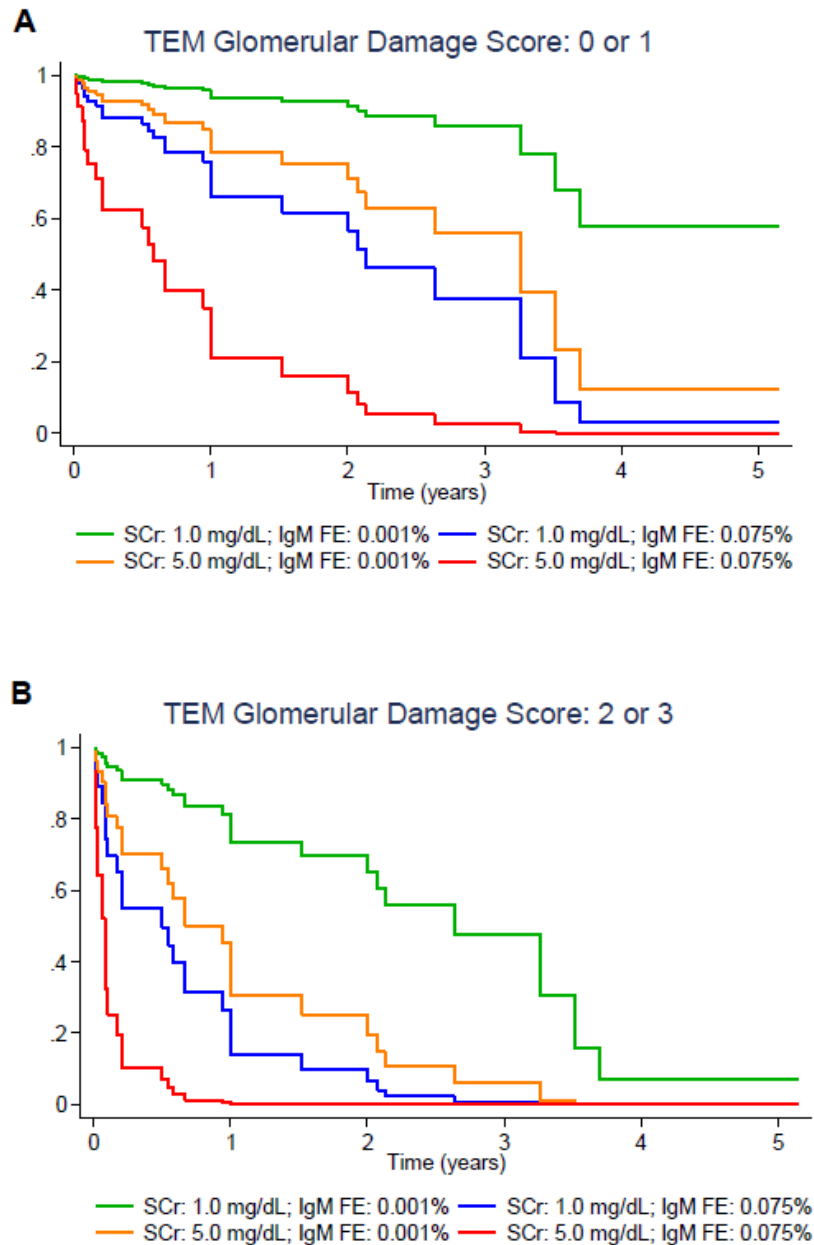


Figure A-1. Probability of survival for dogs with combinations of sCr and IgM_FE at the 25th percentile (1.0 mg/dl and 0.001%, respectively) and 95th percentile (5.0 mg/dl and 0.075%, respectively) based on transmission electron microscopy (TEM) scores: A) TEM score 0/1 and B) TEM score 2/3 (n=60). Abbreviation explanations in Table A-6 and A-9 legends.¹²

APPENDIX C

Supplemental Table for Chapter III

Table A-11. Ability of urinary protein biomarkers to predict ICGN in dogs with proteinuric renal disease that were biopsied for suspicion of glomerular disease. Areas under the curve, optimal cutoff values, and corresponding sensitivities and specificities, as determined by receiver operator characteristic analysis, are displayed.

	All IRIS Stages (N = 178)		IRIS Stage 1 (N = 83)		IRIS Stages 2, 3, 4 (N = 95)	
	AUC	Cutoff (Sens %, Spec %)	AUC	Cutoff (Sens %, Spec %)	AUC	Cutoff (Sens %, Spec %)
UPC	0.6299	>7.0 (58.8, 62.4)	0.5762	>8.9 (35.3, 79.6)	0.6627	>5.3 (74.5, 59.1)
uIgM (ng/ml)	0.8106	>5.3 (83.5, 68.8)	0.7305	>5.3 (79.4, 63.3)	0.8748	>5.1 (86.3, 75.0)
uIgM/c (μg/mg)	0.7880	>10.8 (62.4, 81.7)	0.7527	>6.4 (79.4, 69.4)	0.8124	>14.0 (66.7, 88.6)
IgM_FE (%)	0.7098	>0.007 (48.4, 85.3)	0.6846	>0.002 (69.2, 62.5)	0.7271	>0.008 (71.1, 71.4)
uNAG (U/L)	0.7478	>15.3 (71.8, 65.6)	0.6459	>27.6 (44.1, 87.8)	0.8409	>11.1 (88.2, 68.2)
uNAG/c (U/g)	0.7098	>14.9 (72.9, 60.2)	0.6705	>23.5 (52.9, 79.6)	0.7366	>14.9 (78.4, 63.6)

AUC, area under the curve; ROC, receiver operator characteristic; ICGN, Immune Complex-Mediated Glomerulonephritis; IRIS, International Renal Interest Society; Sens, sensitivity; Spec, specificity; UPC, urine protein:creatinine; uIgM, urine immunoglobulin M; uIgM/c, urine immunoglobulin M/creatinine; IgM_FE, fractional excretion of immunoglobulin M; uNAG, urine N-acetyl-β-D-glucosaminidase; uNAG/c, urine N-acetyl-β-D-glucosaminidase/creatinine.